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(54) Title: NUCLEIC ACID SEQUENCES TO PROTEINS INVOLVED IN TOCOPHEROL SYNTHESIS

(57) Abstract: Nucleic acid sequences and methods are provided for producing plants and seeds having altered tocopherol content and compositions. The methods find particular use in increasing the tocopherol levels in plants, and in providing desirable tocopherol compositions in a host plant cell.

NUCLEIC ACID SEQUENCES TO PROTEINS INVOLVED IN TOCOPHEROL SYNTHESIS

INTRODUCTION

TECHNICAL FIELD

The present invention is directed to nucleic acid and amino acid sequences and constructs, and methods related thereto.

BACKGROUND

Isoprenoids are ubiquitous compounds found in all living organisms. Plants synthesize a diverse array of greater than 22,000 isoprenoids (Connolly and Hill (1992) *Dictionary of Terpenoids*, Chapman and Hall, New York, NY). In plants, isoprenoids play essential roles in particular cell functions such as production of sterols, contributing to eukaryotic membrane architecture, acyclic polyprenoids found in the side chain of ubiquinone and plastoquinone, growth regulators like abscisic acid, gibberellins, brassinosteroids or the photosynthetic pigments chlorophylls and carotenoids. Although the physiological role of other plant isoprenoids is less evident, like that of the vast array of secondary metabolites, some are known to play key roles mediating the adaptative responses to different environmental challenges. In spite of the remarkable diversity of structure and function, all isoprenoids originate from a single metabolic precursor, isopentenyl diphosphate (IPP) (Wright, (1961) *Annu. Rev. Biochem.* 20:525-548; and Spurgeon and Porter, (1981) in Biosynthesis of Isoprenoid Compounds, Porter and Spurgeon eds (John Wiley, New York) Vol. 1, pp1-46).

A number of unique and interconnected biochemical pathways derived from the isoprenoid pathway leading to secondary metabolites, including tocopherols, exist in chloroplasts of higher plants. Tocopherols not only perform vital functions in

plants, but are also important from mammalian nutritional perspectives. In plastids, tocopherols account for up to 40% of the total quinone pool.

Tocopherols and tocotrienols (unsaturated tocopherol derivatives) are well known antioxidants, and play an important role in protecting cells from free radical damage, and in the prevention of many diseases, including cardiac disease, cancer, cataracts, retinopathy, Alzheimer's disease, and neurodegeneration, and have been shown to have beneficial effects on symptoms of arthritis, and in anti-aging. Vitamin E is used in chicken feed for improving the shelf life, appearance, flavor, and oxidative stability of meat, and to transfer tocopherols from feed to eggs. Vitamin E has been shown to be essential for normal reproduction, improves overall performance, and enhances immunocompetence in livestock animals. Vitamin E supplement in animal feed also imparts oxidative stability to milk products.

The demand for natural tocopherols as supplements has been steadily growing at a rate of 10-20% for the past three years. At present, the demand exceeds the supply for natural tocopherols, which are known to be more biopotent than racemic mixtures of synthetically produced tocopherols. Naturally occurring tocopherols are all *d*-stereoisomers, whereas synthetic α -tocopherol is a mixture of eight *d,l*- α -tocopherol isomers, only one of which (12.5%) is identical to the natural *d*- α -tocopherol. Natural *d*- α -tocopherol has the highest vitamin E activity (1.49 IU/mg) when compared to other natural tocopherols or tocotrienols. The synthetic α -tocopherol has a vitamin E activity of 1.1 IU/mg. In 1995, the worldwide market for raw refined tocopherols was \$1020 million; synthetic materials comprised 85-88% of the market, the remaining 12-15% being natural materials. The best sources of natural tocopherols and tocotrienols are vegetable oils and grain products. Currently, most of the natural Vitamin E is produced from γ -tocopherol derived from soy oil processing, which is subsequently converted to α -tocopherol by chemical modification (α -tocopherol exhibits the greatest biological activity).

Methods of enhancing the levels of tocopherols and tocotrienols in plants, especially levels of the more desirable compounds that can be used directly, without

chemical modification, would be useful to the art as such molecules exhibit better functionality and bioavailability.

In addition, methods for the increased production of other isoprenoid derived compounds in a host plant cell is desirable. Furthermore, methods for the production of particular isoprenoid compounds in a host plant cell is also needed.

SUMMARY OF THE INVENTION

The present invention is directed to sequences to proteins involved in tocopherol synthesis. The polynucleotides and polypeptides of the present invention include those derived from prokaryotic and eukaryotic sources.

Thus, one aspect of the present invention relates to prenyltransferase, and in particular to isolated polynucleotide sequences encoding prenyltransferase proteins and polypeptides related thereto. In particular, isolated nucleic acid sequences encoding prenyltransferase proteins from bacterial and plant sources are provided.

In another aspect, the present invention provides isolated polynucleotide sequences encoding tocopherol cyclase, and polypeptides related thereto. In particular, isolated nucleic acid sequences encoding tocopherol cyclase proteins from bacterial and plant sources are provided.

Another aspect of the present invention relates to oligonucleotides which include partial or complete prenyltransferase or tocopherol cyclase encoding sequences.

It is also an aspect of the present invention to provide recombinant DNA constructs which can be used for transcription or transcription and translation (expression) of prenyltransferase or tocopherol cyclase. In particular, constructs are provided which are capable of transcription or transcription and translation in host cells.

In another aspect of the present invention, methods are provided for production of prenyltransferase or tocopherol cyclase in a host cell or progeny thereof. In particular, host cells are transformed or transfected with a DNA construct which can be used for transcription or transcription and translation of

prenyltransferase or tocopherol cyclase. The recombinant cells which contain prenyltransferase or tocopherol cyclase are also part of the present invention.

In a further aspect, the present invention relates to methods of using polynucleotide and polypeptide sequences to modify the tocopherol content of host
5 cells, particularly in host plant cells. Plant cells having such a modified tocopherol content are also contemplated herein. Methods and cells in which both prenyltransferase and tocopherol cyclase are expressed in a host cell are also part of the present invention.

The modified plants, seeds and oils obtained by the expression of the
10 prenyltransferase or tocopherol cyclase are also considered part of the invention.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 provides an amino acid sequence alignment between ATPT2, ATPT3, ATPT4, ATPT8, and ATPT12 are performed using ClustalW.

Figure 2 provides a schematic picture of the expression construct pCGN10800.

15 Figure 3 provides a schematic picture of the expression construct pCGN10801.

Figure 4 provides a schematic picture of the expression construct pCGN10803.

Figure 5 provides a schematic picture of the construct pCGN10806.

Figure 6 provides a schematic picture of the construct pCGN10807.

Figure 7 provides a schematic picture of the construct pCGN10808.

20 Figure 8 provides a schematic picture of the expression construct pCGN10809.

Figure 9 provides a schematic picture of the expression construct pCGN10810.

Figure 10 provides a schematic picture of the expression construct pCGN10811.

Figure 11 provides a schematic picture of the expression construct pCGN10812.

Figure 12 provides a schematic picture of the expression construct pCGN10813.

25 Figure 13 provides a schematic picture of the expression construct pCGN10814.

Figure 14 provides a schematic picture of the expression construct pCGN10815.

Figure 15 provides a schematic picture of the expression construct pCGN10816.

Figure 16 provides a schematic picture of the expression construct pCGN10817.

Figure 17 provides a schematic picture of the expression construct pCGN10819.

Figure 18 provides a schematic picture of the expression construct pCGN10824.

Figure 19 provides a schematic picture of the expression construct pCGN10825.

Figure 20 provides a schematic picture of the expression construct pCGN10826.

Figure 21 provides an amino acid sequence alignment using ClustalW between the
5 *Synechocystis* prenyltransferase sequences.

Figure 22 provides an amino acid sequence of the ATPT2, ATPT3, ATPT4, ATPT8, and ATPT12 protein sequences from *Arabidopsis* and the slr1736, slr0926, slr11899, slr0056, and the slr1518 amino acid sequences from *Synechocystis*.

Figure 23 provides the results of the enzymatic assay from preparations of
10 wild type *Synechocystis* strain 6803, and *Synechocystis* slr1736 knockout.

Figure 24 provides bar graphs of HPLC data obtained from seed extracts of transgenic *Arabidopsis* containing pCGN10822, which provides of the expression of the ATPT2 sequence, in the sense orientation, from the napin promoter. Provided are graphs for alpha, gamma, and delta tocopherols, as well as total tocopherol for 22
15 transformed lines, as well as a nontransformed (wildtype) control.

Figure 25 provides a bar graph of HPLC analysis of seed extracts from *Arabidopsis* plants transformed with pCGN10803 (35S-ATPT2, in the antisense orientation), pCGN10822 (line 1625, napin ATPT2 in the sense orientation), pCGN10809 (line 1627, 35S-ATPT3 in the sense orientation), a nontransformed (wt)
20 control, and an empty vector transformed control.

Figure 26 shows total tocopherol levels measured in T# *Arabidopsis* seed of line.

Figure 27 shows total tocopherol levels measured in T# *Arabidopsis* seed of line.

Figure 28 shows total tocopherol levels measured in developing canola seed of
25 line 10822-1.

Figure 29: shows results of phytyl prenyltransferase activity assay using *Synechocystis* wild type and slr1737 knockout mutant membrane preparations.

Figure 30 is the chromatograph from an HPLC analysis of *Synechocystis* extracts.

Figure 31 is a sequence alignment of the *Arabidopsis* homologue with the sequence of the public database.

5 Figure 32 shows the results of hydropathic analysis of slr1737

Figure 33 shows the results of hydropathic analysis of the *Arabidopsis* homologue of slr1737.

Figure 34 shows the catalytic mechanism of various cyclase enzymes

Figure 35 is a sequence alignment of slr1737, slr1737 *Arabidopsis* homologue
10 and the *Arabidopsis* chalcone isomerase.

DETAILED DESCRIPTION OF THE INVENTION

The present invention provides, *inter alia*, compositions and methods for altering (for example, increasing and decreasing) the tocopherol levels and/or modulating their ratios in host cells. In particular, the present invention provides
15 polynucleotides, polypeptides, and methods of use thereof for the modulation of tocopherol content in host plant cells.

The biosynthesis of α -tocopherol in higher plants involves condensation of homogentisic acid and phytylpyrophosphate to form 2-methyl-6 phytylbenzoquinol that can, by cyclization and subsequent methylations (Fiedler et al., 1982, *Planta*, 155:
20 511-515, Soll et al., 1980, *Arch. Biochem. Biophys.* 204: 544-550, Marshall et al., 1985 *Phytochem.*, 24: 1705-1711, all of which are herein incorporated by reference in their entirety), form various tocopherols.

The *Arabidopsis pds2* mutant identified and characterized by Norris *et al.* (1995), is deficient in tocopherol and plastoquinone-9 accumulation. Further genetic
25 and biochemical analysis suggested that the protein encoded by *PDS2* may be responsible for the prenylation of homogentisic acid. The *PDS2* locus identified by Norris *et al.* (1995) has been hypothesized to possibly encode the tocopherol phytyl-prenyltransferase, as the *pds2* mutant fails to accumulate tocopherols.

Norris *et al.* (1995) determined that in *Arabidopsis pds2* lies at the top of chromosome 3, approximately 7 centimorgans above long hypocotyl2, based on the genetic map. ATPT2 is located on chromosome 2 between 36 and 41 centimorgans, lying on BAC F19F24, indicating that ATPT2 does not correspond to *PDS2*. Thus, it is an aspect of the present invention to provide novel polynucleotides and polypeptides involved in the prenylation of homogentisic acid. This reaction may be a rate limiting step in tocopherol biosynthesis, and this gene has yet to be isolated.

U.S. Patent No. 5,432,069 describes the partial purification and characterization of tocopherol cyclase from *Chlorella protothecoides*, *Dunaliella salina* and wheat. The cyclase described as being glycine rich, water soluble and with a predicted MW of 48-50kDa. However, only limited peptide fragment sequences were available.

In one aspect, the present invention provides polynucleotide and polypeptide sequences involved in the prenylation of straight chain and aromatic compounds. Straight chain prenyltransferases as used herein comprises sequences which encode proteins involved in the prenylation of straight chain compounds, including, but not limited to, geranyl geranyl pyrophosphate and farnesyl pyrophosphate. Aromatic prenyltransferases, as used herein, comprises sequences which encode proteins involved in the prenylation of aromatic compounds, including, but not limited to, menaquinone, ubiquinone, chlorophyll, and homogentisic acid. The prenyltransferase of the present invention preferably prenylates homogentisic acid.

In another aspect, the invention provides polynucleotide and polypeptide sequences to tocopherol cyclization enzymes. The 2,3-dimethyl-5-phytylplastoquinol cyclase (tocopherol cyclase) is responsible for the cyclization of 2,3-dimethyl-5-phytylplastoquinol to tocopherol.

Isolated Polynucleotides, Proteins, and Polypeptides

A first aspect of the present invention relates to isolated prenyltransferase polynucleotides. Another aspect of the present invention relates to isolated tocopherol cyclase polynucleotides. The polynucleotide sequences of the present invention

include isolated polynucleotides that encode the polypeptides of the invention having a deduced amino acid sequence selected from the group of sequences set forth in the Sequence Listing and to other polynucleotide sequences closely related to such sequences and variants thereof.

5 The invention provides a polynucleotide sequence identical over its entire length to each coding sequence as set forth in the Sequence Listing. The invention also provides the coding sequence for the mature polypeptide or a fragment thereof, as well as the coding sequence for the mature polypeptide or a fragment thereof in a reading frame with other coding sequences, such as those encoding a leader or
10 secretory sequence, a pre-, pro-, or prepro- protein sequence. The polynucleotide can also include non-coding sequences, including for example, but not limited to, non-coding 5' and 3' sequences, such as the transcribed, untranslated sequences, termination signals, ribosome binding sites, sequences that stabilize mRNA, introns, polyadenylation signals, and additional coding sequence that encodes additional
15 amino acids. For example, a marker sequence can be included to facilitate the purification of the fused polypeptide. Polynucleotides of the present invention also include polynucleotides comprising a structural gene and the naturally associated sequences that control gene expression.

The invention also includes polynucleotides of the formula:

20 $X-(R_1)_n-(R_2)-(R_3)_n-Y$

wherein, at the 5' end, X is hydrogen, and at the 3' end, Y is hydrogen or a metal, R_1 and R_3 are any nucleic acid residue, n is an integer between 1 and 3000, preferably between 1 and 1000 and R_2 is a nucleic acid sequence of the invention, particularly a nucleic acid sequence selected from the group set forth in the Sequence Listing and
25 preferably those of SEQ ID NOs: 1, 3, 5, 7, 8, 10, 11, 13-16, 18, 23, 29, 36, and 38. In the formula, R_2 is oriented so that its 5' end residue is at the left, bound to R_1 , and its 3' end residue is at the right, bound to R_3 . Any stretch of nucleic acid residues denoted by either R group, where R is greater than 1, may be either a heteropolymer or a homopolymer, preferably a heteropolymer.

The invention also relates to variants of the polynucleotides described herein that encode for variants of the polypeptides of the invention. Variants that are fragments of the polynucleotides of the invention can be used to synthesize full-length polynucleotides of the invention. Preferred embodiments are polynucleotides
5 encoding polypeptide variants wherein 5 to 10, 1 to 5, 1 to 3, 2, 1 or no amino acid residues of a polypeptide sequence of the invention are substituted, added or deleted, in any combination. Particularly preferred are substitutions, additions, and deletions that are silent such that they do not alter the properties or activities of the polynucleotide or polypeptide.

10 Further preferred embodiments of the invention that are at least 50%, 60%, or 70% identical over their entire length to a polynucleotide encoding a polypeptide of the invention, and polynucleotides that are complementary to such polynucleotides. More preferable are polynucleotides that comprise a region that is at least 80% identical over its entire length to a polynucleotide encoding a polypeptide of the
15 invention and polynucleotides that are complementary thereto. In this regard, polynucleotides at least 90% identical over their entire length are particularly preferred, those at least 95% identical are especially preferred. Further, those with at least 97% identity are highly preferred and those with at least 98% and 99% identity are particularly highly preferred, with those at least 99% being the most highly
20 preferred.

Preferred embodiments are polynucleotides that encode polypeptides that retain substantially the same biological function or activity as the mature polypeptides encoded by the polynucleotides set forth in the Sequence Listing.

The invention further relates to polynucleotides that hybridize to the above-
25 described sequences. In particular, the invention relates to polynucleotides that hybridize under stringent conditions to the above-described polynucleotides. As used herein, the terms "stringent conditions" and "stringent hybridization conditions" mean that hybridization will generally occur if there is at least 95% and preferably at least 97% identity between the sequences. An example of stringent hybridization

conditions is overnight incubation at 42°C in a solution comprising 50% formamide, 5x SSC (150 mM NaCl, 15 mM trisodium citrate), 50 mM sodium phosphate (pH 7.6), 5x Denhardt's solution, 10% dextran sulfate, and 20 micrograms/milliliter denatured, sheared salmon sperm DNA, followed by washing the hybridization support in 0.1x SSC at approximately 65°C. Other hybridization and wash conditions are well known and are exemplified in Sambrook, *et al.*, *Molecular Cloning: A Laboratory Manual*, Second Edition, Cold Spring Harbor, NY (1989), particularly Chapter 11.

The invention also provides a polynucleotide consisting essentially of a polynucleotide sequence obtainable by screening an appropriate library containing the complete gene for a polynucleotide sequence set forth in the Sequence Listing under stringent hybridization conditions with a probe having the sequence of said polynucleotide sequence or a fragment thereof; and isolating said polynucleotide sequence. Fragments useful for obtaining such a polynucleotide include, for example, probes and primers as described herein.

As discussed herein regarding polynucleotide assays of the invention, for example, polynucleotides of the invention can be used as a hybridization probe for RNA, cDNA, or genomic DNA to isolate full length cDNAs or genomic clones encoding a polypeptide and to isolate cDNA or genomic clones of other genes that have a high sequence similarity to a polynucleotide set forth in the Sequence Listing. Such probes will generally comprise at least 15 bases. Preferably such probes will have at least 30 bases and can have at least 50 bases. Particularly preferred probes will have between 30 bases and 50 bases, inclusive.

The coding region of each gene that comprises or is comprised by a polynucleotide sequence set forth in the Sequence Listing may be isolated by screening using a DNA sequence provided in the Sequence Listing to synthesize an oligonucleotide probe. A labeled oligonucleotide having a sequence complementary to that of a gene of the invention is then used to screen a library of cDNA, genomic DNA or mRNA to identify members of the library which hybridize to the probe. For

example, synthetic oligonucleotides are prepared which correspond to the prenyltransferase or tocopherol cyclase EST sequences. The oligonucleotides are used as primers in polymerase chain reaction (PCR) techniques to obtain 5' and 3' terminal sequence of prenyltransferase or tocopherol cyclase genes. Alternatively, 5 where oligonucleotides of low degeneracy can be prepared from particular prenyltransferase or tocopherol cyclase peptides, such probes may be used directly to screen gene libraries for prenyltransferase or tocopherol cyclase gene sequences. In particular, screening of cDNA libraries in phage vectors is useful in such methods due to lower levels of background hybridization.

10 Typically, a prenyltransferase or tocopherol cyclase sequence obtainable from the use of nucleic acid probes will show 60-70% sequence identity between the target prenyltransferase or tocopherol cyclase sequence and the encoding sequence used as a probe. However, lengthy sequences with as little as 50-60% sequence identity may also be obtained. The nucleic acid probes may be a lengthy fragment of the nucleic 15 acid sequence, or may also be a shorter, oligonucleotide probe. When longer nucleic acid fragments are employed as probes (greater than about 100 bp), one may screen at lower stringencies in order to obtain sequences from the target sample which have 20-50% deviation (i.e., 50-80% sequence homology) from the sequences used as probe. Oligonucleotide probes can be considerably shorter than the entire nucleic acid 20 sequence encoding an prenyltransferase or tocopherol cyclase enzyme, but should be at least about 10, preferably at least about 15, and more preferably at least about 20 nucleotides. A higher degree of sequence identity is desired when shorter regions are used as opposed to longer regions. It may thus be desirable to identify regions of highly conserved amino acid sequence to design oligonucleotide probes for detecting 25 and recovering other related prenyltransferase or tocopherol cyclase genes. Shorter probes are often particularly useful for polymerase chain reactions (PCR), especially when highly conserved sequences can be identified. (See, Gould, *et al.*, *PNAS USA* (1989) 86:1934-1938.).

Another aspect of the present invention relates to prenyltransferase or tocopherol cyclase polypeptides. Such polypeptides include isolated polypeptides set forth in the Sequence Listing, as well as polypeptides and fragments thereof, particularly those polypeptides which exhibit prenyltransferase or tocopherol cyclase activity and also those polypeptides which have at least 50%, 60% or 70% identity, preferably at least 80% identity, more preferably at least 90% identity, and most preferably at least 95% identity to a polypeptide sequence selected from the group of sequences set forth in the Sequence Listing, and also include portions of such polypeptides, wherein such portion of the polypeptide preferably includes at least 30 amino acids and more preferably includes at least 50 amino acids.

"Identity", as is well understood in the art, is a relationship between two or more polypeptide sequences or two or more polynucleotide sequences, as determined by comparing the sequences. In the art, "identity" also means the degree of sequence relatedness between polypeptide or polynucleotide sequences, as determined by the match between strings of such sequences. "Identity" can be readily calculated by known methods including, but not limited to, those described in *Computational Molecular Biology*, Lesk, A.M., ed., Oxford University Press, New York (1988); *Biocomputing: Informatics and Genome Projects*, Smith, D.W., ed., Academic Press, New York, 1993; *Computer Analysis of Sequence Data, Part I*, Griffin, A.M. and Griffin, H.G., eds., Humana Press, New Jersey (1994); *Sequence Analysis in Molecular Biology*, von Heinje, G., Academic Press (1987); *Sequence Analysis Primer*, Gribskov, M. and Devereux, J., eds., Stockton Press, New York (1991); and Carillo, H., and Lipman, D., *SIAM J Applied Math*, 48:1073 (1988). Methods to determine identity are designed to give the largest match between the sequences tested. Moreover, methods to determine identity are codified in publicly available programs. Computer programs which can be used to determine identity between two sequences include, but are not limited to, GCG (Devereux, J., et al., *Nucleic Acids Research* 12(1):387 (1984); suite of five BLAST programs, three designed for nucleotide sequences queries (BLASTN, BLASTX, and TBLASTX) and two

designed for protein sequence queries (BLASTP and TBLASTN) (Coulson, *Trends in Biotechnology*, 12: 76-80 (1994); Birren, *et al.*, *Genome Analysis*, 1: 543-559 (1997)). The BLAST X program is publicly available from NCBI and other sources (*BLAST Manual*, Altschul, S., *et al.*, NCBI NLM NIH, Bethesda, MD 20894; Altschul, S., *et al.*, *J. Mol. Biol.*, 215:403-410 (1990)). The well known Smith Waterman algorithm can also be used to determine identity.

Parameters for polypeptide sequence comparison typically include the following:

Algorithm: Needleman and Wunsch, *J. Mol. Biol.* 48:443-453 (1970)
 Comparison matrix: BLOSSUM62 from Hentikoff and Hentikoff, *Proc. Natl. Acad. Sci USA* 89:10915-10919 (1992)

Gap Penalty: 12

Gap Length Penalty: 4

A program which can be used with these parameters is publicly available as the "gap" program from Genetics Computer Group, Madison Wisconsin. The above parameters along with no penalty for end gap are the default parameters for peptide comparisons.

Parameters for polynucleotide sequence comparison include the following:

Algorithm: Needleman and Wunsch, *J. Mol. Biol.* 48:443-453 (1970)

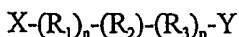
Comparison matrix: matches = +10; mismatches = 0

Gap Penalty: 50

Gap Length Penalty: 3

A program which can be used with these parameters is publicly available as the "gap" program from Genetics Computer Group, Madison Wisconsin. The above parameters are the default parameters for nucleic acid comparisons.

The invention also includes polypeptides of the formula:



wherein, at the amino terminus, X is hydrogen, and at the carboxyl terminus, Y is hydrogen or a metal, R₁ and R₃ are any amino acid residue, n is an integer between 1

and 1000, and R_2 is an amino acid sequence of the invention, particularly an amino acid sequence selected from the group set forth in the Sequence Listing and preferably those encoded by the sequences provided in SEQ ID NOs: 2, 4, 6, 9, 12, 17, 19-22, 24-28, 30, 32-35, 37, and 39. In the formula, R_2 is oriented so that its amino terminal residue is at the left, bound to R_1 , and its carboxy terminal residue is at the right, bound to R_3 . Any stretch of amino acid residues denoted by either R group, where R is greater than 1, may be either a heteropolymer or a homopolymer, preferably a heteropolymer.

Polypeptides of the present invention include isolated polypeptides encoded by a polynucleotide comprising a sequence selected from the group of a sequence contained in the Sequence Listing set forth herein .

The polypeptides of the present invention can be mature protein or can be part of a fusion protein.

Fragments and variants of the polypeptides are also considered to be a part of the invention. A fragment is a variant polypeptide which has an amino acid sequence that is entirely the same as part but not all of the amino acid sequence of the previously described polypeptides. The fragments can be "free-standing" or comprised within a larger polypeptide of which the fragment forms a part or a region, most preferably as a single continuous region. Preferred fragments are biologically active fragments which are those fragments that mediate activities of the polypeptides of the invention, including those with similar activity or improved activity or with a decreased activity. Also included are those fragments that antigenic or immunogenic in an animal, particularly a human.

Variants of the polypeptide also include polypeptides that vary from the sequences set forth in the Sequence Listing by conservative amino acid substitutions, substitution of a residue by another with like characteristics. In general, such substitutions are among Ala, Val, Leu and Ile; between Ser and Thr; between Asp and Glu; between Asn and Gln; between Lys and Arg; or between Phe and Tyr.

Particularly preferred are variants in which 5 to 10; 1 to 5; 1 to 3 or one amino acid(s) are substituted, deleted, or added, in any combination.

5 Variants that are fragments of the polypeptides of the invention can be used to produce the corresponding full length polypeptide by peptide synthesis. Therefore, these variants can be used as intermediates for producing the full-length polypeptides of the invention.

The polynucleotides and polypeptides of the invention can be used, for example, in the transformation of host cells, such as plant host cells, as further discussed herein.

10 The invention also provides polynucleotides that encode a polypeptide that is a mature protein plus additional amino or carboxyl-terminal amino acids, or amino acids within the mature polypeptide (for example, when the mature form of the protein has more than one polypeptide chain). Such sequences can, for example, play a role in the processing of a protein from a precursor to a mature form, allow protein
15 transport, shorten or lengthen protein half-life, or facilitate manipulation of the protein in assays or production. It is contemplated that cellular enzymes can be used to remove any additional amino acids from the mature protein.

A precursor protein, having the mature form of the polypeptide fused to one or more prosequences may be an inactive form of the polypeptide. The inactive
20 precursors generally are activated when the prosequences are removed. Some or all of the prosequences may be removed prior to activation. Such precursor protein are generally called proproteins.

Plant Constructs and Methods of Use

Of particular interest is the use of the nucleotide sequences in recombinant
25 DNA constructs to direct the transcription or transcription and translation (expression) of the prenyltransferase or tocopherol cyclase sequences of the present invention in a host plant cell. The expression constructs generally comprise a promoter functional in a host plant cell operably linked to a nucleic acid sequence encoding a

prenyltransferase or tocopherol cyclase of the present invention and a transcriptional termination region functional in a host plant cell.

A first nucleic acid sequence is "operably linked" or "operably associated" with a second nucleic acid sequence when the sequences are so arranged that the first
5 nucleic acid sequence affects the function of the second nucleic acid sequence. Preferably, the two sequences are part of a single contiguous nucleic acid molecule and more preferably are adjacent. For example, a promoter is operably linked to a gene if the promoter regulates or mediates transcription of the gene in a cell.

Those skilled in the art will recognize that there are a number of promoters
10 which are functional in plant cells, and have been described in the literature. Chloroplast and plastid specific promoters, chloroplast or plastid functional promoters, and chloroplast or plastid operable promoters are also envisioned.

One set of plant functional promoters are constitutive promoters such as the CaMV35S or FMV35S promoters that yield high levels of expression in most plant
15 organs. Enhanced or duplicated versions of the CaMV35S and FMV35S promoters are useful in the practice of this invention (Odell, *et al.* (1985) *Nature* 313:810-812; Rogers, U.S. Patent Number 5,378, 619). In addition, it may also be preferred to bring about expression of the prenyltransferase or tocopherol cyclase gene in specific tissues of the plant, such as leaf, stem, root, tuber, seed, fruit, etc., and the promoter chosen
20 should have the desired tissue and developmental specificity.

Of particular interest is the expression of the nucleic acid sequences of the present invention from transcription initiation regions which are preferentially expressed in a plant seed tissue. Examples of such seed preferential transcription initiation sequences include those sequences derived from sequences encoding plant
25 storage protein genes or from genes involved in fatty acid biosynthesis in oilseeds. Examples of such promoters include the 5' regulatory regions from such genes as napin (Kridl *et al.*, *Seed Sci. Res.* 1:209:219 (1991)), phaseolin, zein, soybean trypsin inhibitor, ACP, stearyl-ACP desaturase, soybean α' subunit of β -conglycinin (soy 7s, (Chen *et al.*, *Proc. Natl. Acad. Sci.*, 83:8560-8564 (1986))) and oleosin.

It may be advantageous to direct the localization of proteins conferring prenyltransferase or tocopherol cyclase to a particular subcellular compartment, for example, to the mitochondrion, endoplasmic reticulum, vacuoles, chloroplast or other plastidic compartment. For example, where the genes of interest of the present invention will be targeted to plastids, such as chloroplasts, for expression, the constructs will also employ the use of sequences to direct the gene to the plastid. Such sequences are referred to herein as chloroplast transit peptides (CTP) or plastid transit peptides (PTP). In this manner, where the gene of interest is not directly inserted into the plastid, the expression construct will additionally contain a gene encoding a transit peptide to direct the gene of interest to the plastid. The chloroplast transit peptides may be derived from the gene of interest, or may be derived from a heterologous sequence having a CTP. Such transit peptides are known in the art. See, for example, Von Heijne *et al.* (1991) *Plant Mol. Biol. Rep.* 9:104-126; Clark *et al.* (1989) *J. Biol. Chem.* 264:17544-17550; della-Cioppa *et al.* (1987) *Plant Physiol.* 84:965-968; Romer *et al.* (1993) *Biochem. Biophys. Res Commun.* 196:1414-1421; and, Shah *et al.* (1986) *Science* 233:478-481.

Depending upon the intended use, the constructs may contain the nucleic acid sequence which encodes the entire prenyltransferase or tocopherol cyclase protein, or a portion thereof. For example, where antisense inhibition of a given prenyltransferase or tocopherol cyclase protein is desired, the entire prenyltransferase or tocopherol cyclase sequence is not required. Furthermore, where prenyltransferase or tocopherol cyclase sequences used in constructs are intended for use as probes, it may be advantageous to prepare constructs containing only a particular portion of a prenyltransferase or tocopherol cyclase encoding sequence, for example a sequence which is discovered to encode a highly conserved prenyltransferase or tocopherol cyclase region.

The skilled artisan will recognize that there are various methods for the inhibition of expression of endogenous sequences in a host cell. Such methods include, but are not limited to, antisense suppression (Smith, *et al.* (1988) *Nature*

334:724-726) , co-suppression (Napoli, *et al.* (1989) *Plant Cell* 2:279-289),
ribozymes (PCT Publication WO 97/10328), and combinations of sense and antisense
Waterhouse, *et al.* (1998) *Proc. Natl. Acad. Sci. USA* 95:13959-13964. Methods for
the suppression of endogenous sequences in a host cell typically employ the
5 transcription or transcription and translation of at least a portion of the sequence to be
suppressed. Such sequences may be homologous to coding as well as non-coding
regions of the endogenous sequence.

Regulatory transcript termination regions may be provided in plant expression
constructs of this invention as well. Transcript termination regions may be provided
10 by the DNA sequence encoding the prenyltransferase or tocopherol cyclase or a
convenient transcription termination region derived from a different gene source, for
example, the transcript termination region which is naturally associated with the
transcript initiation region. The skilled artisan will recognize that any convenient
transcript termination region which is capable of terminating transcription in a plant
15 cell may be employed in the constructs of the present invention.

Alternatively, constructs may be prepared to direct the expression of the
prenyltransferase or tocopherol cyclase sequences directly from the host plant cell
plastid. Such constructs and methods are known in the art and are generally
described, for example, in Svab, *et al.* (1990) *Proc. Natl. Acad. Sci. USA* 87:8526-
20 8530 and Svab and Maliga (1993) *Proc. Natl. Acad. Sci. USA* 90:913-917 and in U.S.
Patent Number 5,693,507.

The prenyltransferase or tocopherol cyclase constructs of the present invention
can be used in transformation methods with additional constructs providing for the
expression of other nucleic acid sequences encoding proteins involved in the
25 production of tocopherols, or tocopherol precursors such as homogentisic acid and/or
phytylpyrophosphate. Nucleic acid sequences encoding proteins involved in the
production of homogentisic acid are known in the art, and include but not are limited
to, 4-hydroxyphenylpyruvate dioxygenase (HPPD, EC 1.13.11.27) described for
example, by Garcia, *et al.* ((1999) *Plant Physiol.* 119(4):1507-1516), mono or

bifunctional *tyrA* (described for example by Xia, *et al.* (1992) *J. Gen. Microbiol.* 138:1309-1316, and Hudson, *et al.* (1984) *J. Mol. Biol.* 180:1023-1051), Oxygenase, 4-hydroxyphenylpyruvate di- (9CI), 4-Hydroxyphenylpyruvate dioxygenase; p-Hydroxyphenylpyruvate dioxygenase; p-Hydroxyphenylpyruvate hydroxylase; 5 p-Hydroxyphenylpyruvate oxidase; p-Hydroxyphenylpyruvic acid hydroxylase; p-Hydroxyphenylpyruvic hydroxylase; p-Hydroxyphenylpyruvic oxidase), 4-hydroxyphenylacetate, NAD(P)H: oxygen oxidoreductase (1-hydroxylating); 4-hydroxyphenylacetate 1-monooxygenase, and the like. In addition, constructs for the expression of nucleic acid sequences encoding proteins involved in the production of phytylpyrophosphate can also be employed with the prenyltransferase or tocopherol cyclase constructs of the present invention. Nucleic acid sequences encoding proteins involved in the production of phytylpyrophosphate are known in the art, and include, but are not limited to geranylgeranylpyrophosphate synthase (GGPPS), geranylgeranylpyrophosphate reductase (GGH), 1-deoxyxylulose-5-phosphate 15 synthase, 1- deoxy-D-xylulose-5-phosphate reductoisomerase, 4-diphosphocytidyl-2-C-methylerythritol synthase, isopentyl pyrophosphate isomerase.

The prenyltransferase or tocopherol cyclase sequences of the present invention find use in the preparation of transformation constructs having a second expression cassette for the expression of additional sequences involved in tocopherol 20 biosynthesis. Additional tocopherol biosynthesis sequences of interest in the present invention include, but are not limited to gamma-tocopherol methyltransferase (Shintani, *et al.* (1998) *Science* 282(5396):2098-2100), tocopherol cyclase, and tocopherol methyltransferase.

A plant cell, tissue, organ, or plant into which the recombinant DNA 25 constructs containing the expression constructs have been introduced is considered transformed, transfected, or transgenic. A transgenic or transformed cell or plant also includes progeny of the cell or plant and progeny produced from a breeding program employing such a transgenic plant as a parent in a cross and exhibiting an altered

phenotype resulting from the presence of a prenyltransferase or tocopherol cyclase nucleic acid sequence.

Plant expression or transcription constructs having a prenyltransferase or tocopherol cyclase as the DNA sequence of interest for increased or decreased expression thereof may be employed with a wide variety of plant life, particularly, plant life involved in the production of vegetable oils for edible and industrial uses. Particularly preferred plants for use in the methods of the present invention include, but are not limited to: *Acacia*, alfalfa, aneth, apple, apricot, artichoke, arugula, asparagus, avocado, banana, barley, beans, beet, blackberry, blueberry, broccoli, brussels sprouts, cabbage, canola, cantaloupe, carrot, cassava, cauliflower, celery, cherry, chicory, cilantro, citrus, clementines, coffee, corn, cotton, cucumber, Douglas fir, eggplant, endive, escarole, eucalyptus, fennel, figs, garlic, gourd, grape, grapefruit, honey dew, jicama, kiwifruit, lettuce, leeks, lemon, lime, Loblolly pine, mango, melon, mushroom, nectarine, nut, oat, oil palm, oil seed rape, okra, onion, orange, an ornamental plant, papaya, parsley, pea, peach, peanut, pear, pepper, persimmon, pine, pineapple, plantain, plum, pomegranate, poplar, potato, pumpkin, quince, radiata pine, radicchio, radish, raspberry, rice, rye, sorghum, Southern pine, soybean, spinach, squash, strawberry, sugarbeet, sugarcane, sunflower, sweet potato, sweetgum, tangerine, tea, tobacco, tomato, triticale, turf, turnip, a vine, watermelon, wheat, yams, and zucchini.

Most especially preferred are temperate oilseed crops. Temperate oilseed crops of interest include, but are not limited to, rapeseed (Canola and High Erucic Acid varieties), sunflower, safflower, cotton, soybean, peanut, coconut and oil palms, and corn. Depending on the method for introducing the recombinant constructs into the host cell, other DNA sequences may be required. Importantly, this invention is applicable to dicotyledons and monocotyledons species alike and will be readily applicable to new and/or improved transformation and regulation techniques.

Of particular interest, is the use of prenyltransferase or tocopherol cyclase constructs in plants to produce plants or plant parts, including, but not limited to

leaves, stems, roots, reproductive, and seed, with a modified content of tocopherols in plant parts having transformed plant cells.

For immunological screening, antibodies to the protein can be prepared by injecting rabbits or mice with the purified protein or portion thereof, such methods of preparing antibodies being well known to those in the art. Either monoclonal or polyclonal antibodies can be produced, although typically polyclonal antibodies are more useful for gene isolation. Western analysis may be conducted to determine that a related protein is present in a crude extract of the desired plant species, as determined by cross-reaction with the antibodies to the encoded proteins. When cross-reactivity is observed, genes encoding the related proteins are isolated by screening expression libraries representing the desired plant species. Expression libraries can be constructed in a variety of commercially available vectors, including lambda gt11, as described in Sambrook, *et al.* (*Molecular Cloning: A Laboratory Manual*, Second Edition (1989) Cold Spring Harbor Laboratory, Cold Spring Harbor, New York).

To confirm the activity and specificity of the proteins encoded by the identified nucleic acid sequences as prenyltransferase or tocopherol cyclase enzymes, *in vitro* assays are performed in insect cell cultures using baculovirus expression systems. Such baculovirus expression systems are known in the art and are described by Lee, *et al.* U.S. Patent Number 5,348,886, the entirety of which is herein incorporated by reference.

In addition, other expression constructs may be prepared to assay for protein activity utilizing different expression systems. Such expression constructs are transformed into yeast or prokaryotic host and assayed for prenyltransferase or tocopherol cyclase activity. Such expression systems are known in the art and are readily available through commercial sources.

In addition to the sequences described in the present invention, DNA coding sequences useful in the present invention can be derived from algae, fungi, bacteria, mammalian sources, plants, etc. Homology searches in existing databases using

signature sequences corresponding to conserved nucleotide and amino acid sequences of prenyltransferase or tocopherol cyclase can be employed to isolate equivalent, related genes from other sources such as plants and microorganisms. Searches in EST databases can also be employed. Furthermore, the use of DNA sequences encoding enzymes functionally enzymatically equivalent to those disclosed herein, wherein such DNA sequences are degenerate equivalents of the nucleic acid sequences disclosed herein in accordance with the degeneracy of the genetic code, is also encompassed by the present invention. Demonstration of the functionality of coding sequences identified by any of these methods can be carried out by complementation of mutants of appropriate organisms, such as *Synechocystis*, *Shewanella*, yeast, *Pseudomonas*, *Rhodobacteria*, etc., that lack specific biochemical reactions, or that have been mutated. The sequences of the DNA coding regions can be optimized by gene resynthesis, based on codon usage, for maximum expression in particular hosts.

For the alteration of tocopherol production in a host cell, a second expression construct can be used in accordance with the present invention. For example, the prenyltransferase or tocopherol cyclase expression construct can be introduced into a host cell in conjunction with a second expression construct having a nucleotide sequence for a protein involved in tocopherol biosynthesis.

The method of transformation in obtaining such transgenic plants is not critical to the instant invention, and various methods of plant transformation are currently available. Furthermore, as newer methods become available to transform crops, they may also be directly applied hereunder. For example, many plant species naturally susceptible to *Agrobacterium* infection may be successfully transformed via tripartite or binary vector methods of *Agrobacterium* mediated transformation. In many instances, it will be desirable to have the construct bordered on one or both sides by T-DNA, particularly having the left and right borders, more particularly the right border. This is particularly useful when the construct uses *A. tumefaciens* or *A. rhizogenes* as a mode for transformation, although the T-DNA borders may find use

with other modes of transformation. In addition, techniques of microinjection, DNA particle bombardment, and electroporation have been developed which allow for the transformation of various monocot and dicot plant species.

Normally, included with the DNA construct will be a structural gene having
5 the necessary regulatory regions for expression in a host and providing for selection of transformant cells. The gene may provide for resistance to a cytotoxic agent, e.g. antibiotic, heavy metal, toxin, etc., complementation providing prototrophy to an auxotrophic host, viral immunity or the like. Depending upon the number of different host species the expression construct or components thereof are introduced, one or
10 more markers may be employed, where different conditions for selection are used for the different hosts.

Where *Agrobacterium* is used for plant cell transformation, a vector may be used which may be introduced into the *Agrobacterium* host for homologous recombination with T-DNA or the Ti- or Ri-plasmid present in the *Agrobacterium*
15 host. The Ti- or Ri-plasmid containing the T-DNA for recombination may be armed (capable of causing gall formation) or disarmed (incapable of causing gall formation), the latter being permissible, so long as the *vir* genes are present in the transformed *Agrobacterium* host. The armed plasmid can give a mixture of normal plant cells and gall.

20 In some instances where *Agrobacterium* is used as the vehicle for transforming host plant cells, the expression or transcription construct bordered by the T-DNA border region(s) will be inserted into a broad host range vector capable of replication in *E. coli* and *Agrobacterium*, there being broad host range vectors described in the literature. Commonly used is pRK2 or derivatives thereof. See, for example, Ditta, *et al.*, (Proc. Nat. Acad. Sci., U.S.A. (1980) 77:7347-7351) and EPA 0 120 515, which
25 are incorporated herein by reference. Alternatively, one may insert the sequences to be expressed in plant cells into a vector containing separate replication sequences, one of which stabilizes the vector in *E. coli*, and the other in *Agrobacterium*. See, for example, McBride, *et al.* (Plant Mol. Biol. (1990) 14:269-276), wherein the pRiHRI

(Jouanin, *et al.*, *Mol. Gen. Genet.* (1985) 201:370-374) origin of replication is utilized and provides for added stability of the plant expression vectors in host *Agrobacterium* cells.

Included with the expression construct and the T-DNA will be one or more
5 markers, which allow for selection of transformed *Agrobacterium* and transformed plant cells. A

number of markers have been developed for use with plant cells, such as resistance to chloramphenicol, kanamycin, the aminoglycoside G418, hygromycin, or the like. The particular marker employed is not essential to this invention, one or another marker
10 being preferred depending on the particular host and the manner of construction.

For transformation of plant cells using *Agrobacterium*, explants may be combined and incubated with the transformed *Agrobacterium* for sufficient time for transformation, the bacteria killed, and the plant cells cultured in an appropriate selective medium. Once callus forms, shoot formation can be encouraged by
15 employing the appropriate plant hormones in accordance with known methods and the shoots transferred to rooting medium for regeneration of plants. The plants may then be grown to seed and the seed used to establish repetitive generations and for isolation of vegetable oils.

There are several possible ways to obtain the plant cells of this invention
20 which contain multiple expression constructs. Any means for producing a plant comprising a construct having a DNA sequence encoding the expression construct of the present invention, and at least one other construct having another DNA sequence encoding an enzyme are encompassed by the present invention. For example, the expression construct can be used to transform a plant at the same time as the second
25 construct either by inclusion of both expression constructs in a single transformation vector or by using separate vectors, each of which express desired genes. The second construct can be introduced into a plant which has already been transformed with the prenyltransferase or tocopherol cyclase expression construct, or alternatively, transformed plants, one expressing the prenyltransferase or tocopherol cyclase

construct and one expressing the second construct, can be crossed to bring the constructs together in the same plant.

Transgenic plants of the present invention may be produced from tissue culture, and subsequent generations grown from seed. Alternatively, transgenic plants
5 may be grown using apomixis. Apomixis is a genetically controlled method of reproduction in plants where the embryo is formed without union of an egg and a sperm. There are three basic types of apomictic reproduction: 1) apospory where the embryo develops from a chromosomally unreduced egg in an embryo sac derived from the nucleus, 2) diplospory where the embryo develops from an unreduced egg in
10 an embryo sac derived from the megaspore mother cell, and 3) adventitious embryony where the embryo develops directly from a somatic cell. In most forms of apomixis, pseudogamy or fertilization of the polar nuclei to produce endosperm is necessary for seed viability. In apospory, a nurse cultivar can be used as a pollen source for endosperm formation in seeds. The nurse cultivar does not affect the genetics of the
15 aposporous apomictic cultivar since the unreduced egg of the cultivar develops parthenogenetically, but makes possible endosperm production. Apomixis is economically important, especially in transgenic plants, because it causes any genotype, no matter how heterozygous, to breed true. Thus, with apomictic reproduction, heterozygous transgenic plants can maintain their genetic fidelity
20 throughout repeated life cycles. Methods for the production of apomictic plants are known in the art. See, U.S. Patent No. 5,811,636, which is herein incorporated by reference in its entirety.

The nucleic acid sequences of the present invention can be used in constructs to provide for the expression of the sequence in a variety of host cells, both
25 prokaryotic eukaryotic. Host cells of the present invention preferably include monocotyledenous and dicotyledenous plant cells.

In general, the skilled artisan is familiar with the standard resource materials which describe specific conditions and procedures for the construction, manipulation and isolation of macromolecules (e.g., DNA molecules, plasmids, etc.), generation of

recombinant organisms and the screening and isolating of clones, (see for example, Sambrook *et al.*, *Molecular Cloning: A Laboratory Manual*, Cold Spring Harbor Press (1989); Maliga *et al.*, *Methods in Plant Molecular Biology*, Cold Spring Harbor Press (1995), the entirety of which is herein incorporated by reference; Birren *et al.*,
5 *Genome Analysis: Analyzing DNA*, 1, Cold Spring Harbor, New York, the entirety of which is herein incorporated by reference).

Methods for the expression of sequences in insect host cells are known in the art. Baculovirus expression vectors are recombinant insect viruses in which the coding sequence for a chosen foreign gene has been inserted behind a baculovirus promoter
10 in place of the viral gene, e.g., polyhedrin (Smith and Summers, U.S. Pat. No., 4,745,051, the entirety of which is incorporated herein by reference). Baculovirus expression vectors are known in the art, and are described for example in Doerfler, *Curr. Top. Microbiol. Immunol.* 131:51-68 (1968); Luckow and Summers, *Bio/Technology* 6:47-55 (1988a); Miller, *Annual Review of Microbiol.* 42:177-199
15 (1988); Summers, *Curr. Comm. Molecular Biology*, Cold Spring Harbor Press, Cold Spring Harbor, N.Y. (1988); Summers and Smith, *A Manual of Methods for Baculovirus Vectors and Insect Cell Culture Procedures*, Texas Ag. Exper. Station Bulletin No. 1555 (1988), the entireties of which is herein incorporated by reference)

Methods for the expression of a nucleic acid sequence of interest in a fungal
20 host cell are known in the art. The fungal host cell may, for example, be a yeast cell or a filamentous fungal cell. Methods for the expression of DNA sequences of interest in yeast cells are generally described in "Guide to yeast genetics and molecular biology", Guthrie and Fink, eds. *Methods in enzymology*, Academic Press, Inc. Vol 194 (1991) and *Gene expression technology*", Goeddel ed, *Methods in Enzymology*, Academic
25 Press, Inc., Vol 185 (1991).

Mammalian cell lines available as hosts for expression are known in the art and include many immortalized cell lines available from the American Type Culture Collection (ATCC, Manassas, VA), such as HeLa cells, Chinese hamster ovary (CHO) cells, baby hamster kidney (BHK) cells and a number of other cell lines.

Suitable promoters for mammalian cells are also known in the art and include, but are not limited to, viral promoters such as that from Simian Virus 40 (SV40) (Fiers *et al.*, *Nature* 273:113 (1978), the entirety of which is herein incorporated by reference), Rous sarcoma virus (RSV), adenovirus (ADV) and bovine papilloma virus (BPV).

5 Mammalian cells may also require terminator sequences and poly-A addition sequences. Enhancer sequences which increase expression may also be included and sequences which promote amplification of the gene may also be desirable (for example methotrexate resistance genes).

Vectors suitable for replication in mammalian cells are well known in the art,
10 and may include viral replicons, or sequences which insure integration of the appropriate sequences encoding epitopes into the host genome. Plasmid vectors that greatly facilitate the construction of recombinant viruses have been described (*see*, for example, Mackett *et al.*, *J Virol.* 49:857 (1984); Chakrabarti *et al.*, *Mol. Cell. Biol.* 5:3403 (1985); Moss, In: *Gene Transfer Vectors For Mammalian Cells* (Miller and
15 Calos, eds., Cold Spring Harbor Laboratory, N.Y., p. 10, (1987); all of which are herein incorporated by reference in their entirety).

The invention also includes plants and plant parts, such as seed, oil and meal derived from seed, and feed and food products processed from plants, which are enriched in tocopherols. Of particular interest is seed oil obtained from transgenic
20 plants where the tocopherol level has been increased as compared to seed oil of a non-transgenic plant.

The harvested plant material may be subjected to additional processing to further enrich the tocopherol content. The skilled artisan will recognize that there are many such processes or methods for refining, bleaching and degumming oil. United
25 States Patent Number 5,932,261, issued August 3, 1999, discloses on such process, for the production of a natural carotene rich refined and deodorised oil by subjecting the oil to a pressure of less than 0.060 mbar and to a temperature of less than 200.degree. C. Oil distilled by this process has reduced free fatty acids, yielding a refined, deodorised oil where Vitamin E contained in the feed oil is substantially

retained in the processed oil. The teachings of this patent are incorporated herein by reference.

The invention now being generally described, it will be more readily
5 understood by reference to the following examples which are included for purposes of illustration only and are not intended to limit the present invention.

EXAMPLES

Example 1: Identification of Prenyltransferase or tocopherol cyclase Sequences

10 PSI-BLAST (Altschul, *et al.* (1997) *Nuc Acid Res* 25:3389-3402) profiles were generated for both the straight chain and aromatic classes of prenyltransferases. To generate the straight chain profile, a prenyl- transferase from *Porphyr**a purpurea* (Genbank accession 1709766) was used as a query against the NCBI non-redundant protein database. The *E. coli* enzyme involved in the formation of ubiquinone, ubiA
15 (genbank accession 1790473) was used as a starting sequence to generate the aromatic prenyltransferase profile. These profiles were used to search public and proprietary DNA and protein data bases. In *Arabidopsis* six putative prenyltransferases of the straight-chain class were identified, ATPT1, (SEQ ID NO:9), ATPT7 (SEQ ID NO:10), ATPT8 (SEQ ID NO:11), ATPT9 (SEQ ID NO:13), ATPT10 (SEQ ID
20 NO:14), and ATPT11 (SEQ ID NO:15), and six were identified of the aromatic class, ATPT2 (SEQ ID NO:1), ATPT3 (SEQ ID NO:3), ATPT4 (SEQ ID NO:5), ATPT5 (SEQ ID NO:7), ATPT6 (SEQ ID NO:8), and ATPT12 (SEQ ID NO:16). Additional prenyltransferase sequences from other plants related to the aromatic class of prenyltransferases, such as soy (SEQ ID NOs: 19-23, the deduced amino acid
25 sequence of SEQ ID NO:23 is provided in SEQ ID NO:24) and maize (SEQ ID NOs:25-29, and 31) are also identified. The deduced amino acid sequence of ZMPT5 (SEQ ID NO:29) is provided in SEQ ID NO:30.

Searches are performed on a Silicon Graphics Unix computer using additional Bioaccelerator hardware and GenWeb software supplied by Compugen Ltd. This

software and hardware enables the use of the Smith-Waterman algorithm in searching DNA and protein databases using profiles as queries. The program used to query protein databases is profilesearch. This is a search where the query is not a single sequence but a profile based on a multiple alignment of amino acid or nucleic acid sequences. The profile is used to query a sequence data set, i.e., a sequence database. The profile contains all the pertinent information for scoring each position in a sequence, in effect replacing the "scoring matrix" used for the standard query searches. The program used to query nucleotide databases with a protein profile is tprofilesearch. Tprofilesearch searches nucleic acid databases using an amino acid profile query. As the search is running, sequences in the database are translated to amino acid sequences in six reading frames. The output file for tprofilesearch is identical to the output file for profilesearch except for an additional column that indicates the frame in which the best alignment occurred.

The Smith-Waterman algorithm, (Smith and Waterman (1981) *supra*), is used to search for similarities between one sequence from the query and a group of sequences contained in the database. E score values as well as other sequence information, such as conserved peptide sequences are used to identify related sequences.

To obtain the entire coding region corresponding to the *Arabidopsis* prenyltransferase sequences, synthetic oligo-nucleotide primers are designed to amplify the 5' and 3' ends of partial cDNA clones containing prenyltransferase sequences. Primers are designed according to the respective *Arabidopsis* prenyltransferase sequences and used in Rapid Amplification of cDNA Ends (RACE) reactions (Frohman *et al.* (1988) *Proc. Natl. Acad. Sci. USA* 85:8998-9002) using the Marathon cDNA amplification kit (Clontech Laboratories Inc, Palo Alto, CA).

Amino acid sequence alignments between ATPT2 (SEQ ID NO:2), ATPT3 (SEQ ID NO:4), ATPT4 (SEQ ID NO:6), ATPT8 (SEQ ID NO:12), and ATPT12 (SEQ ID NO:17) are performed using ClustalW (Figure 1), and the percent identity and similarities are provided in Table 1 below.

Table 1:

	ATPT2	ATPT3	ATPT4	ATPT8	ATPT12
ATPT2 % Identity	12	13	11	15	
% similar	25	25	22	32	
% Gap	17	20	20	9	
ATPT3 % Identity		12	6	22	
% similar		29	16	38	
% Gap		20	24	14	
ATPT4 % Identity			9	14	
% similar			18	29	
% Gap			26	19	
ATPT8 % Identity				7	
% similar				19	
% Gap				20	
ATPT12 % Identity					
% similar					
% Gap					

Example 2: Preparation of Prenyl Transferase Expression Constructs

- 5 A plasmid containing the napin cassette derived from pCGN3223 (described in USPN 5,639,790, the entirety of which is incorporated herein by reference) was modified to make it more useful for cloning large DNA fragments containing multiple restriction sites, and to allow the cloning of multiple napin fusion genes into plant binary transformation vectors. An adapter comprised of the self annealed
- 10 oligonucleotide of sequence
- CGCGATTTAAATGGCGCGCCCTGCAGGCGGCCGCCTGCAGGGCGCGCCAT
- TAAAT (SEQ ID NO:40) was ligated into the cloning vector pBC SK+ (Stratagene) after digestion with the restriction endonuclease BssHII to construct vector

pCGN7765. Plasmids pCGN3223 and pCGN7765 were digested with NotI and ligated together. The resultant vector, pCGN7770, contains the pCGN7765 backbone with the napin seed specific expression cassette from pCGN3223.

The cloning cassette, pCGN7787, essentially the same regulatory elements as pCGN7770, with the exception of the napin regulatory regions of pCGN7770 have been replaced with the double CAMV 35S promoter and the tml polyadenylation and transcriptional termination region.

A binary vector for plant transformation, pCGN5139, was constructed from pCGN1558 (McBride and Summerfelt, (1990) Plant Molecular Biology, 14:269-276). The polylinker of pCGN1558 was replaced as a HindIII/Asp718 fragment with a polylinker containing unique restriction endonuclease sites, AscI, PacI, XbaI, SmaI, BamHI, and NotI. The Asp718 and HindIII restriction endonuclease sites are retained in pCGN5139.

A series of turbo binary vectors are constructed to allow for the rapid cloning of DNA sequences into binary vectors containing transcriptional initiation regions (promoters) and transcriptional termination regions.

The plasmid pCGN8618 was constructed by ligating oligonucleotides 5'-TCGAGGATCCGCGGCCGCAAGCTTCCTGCAGG-3' (SEQ ID NO:41) and 5'-TCGACCTGCAGGAAGCTTGCGGCCGCGGATCC-3' (SEQ ID NO:42) into SalI/XhoI-digested pCGN7770. A fragment containing the napin promoter, polylinker and napin 3' region was excised from pCGN8618 by digestion with Asp718I; the fragment was blunt-ended by filling in the 5' overhangs with Klenow fragment then ligated into pCGN5139 that had been digested with Asp718I and HindIII and blunt-ended by filling in the 5' overhangs with Klenow fragment. A plasmid containing the insert oriented so that the napin promoter was closest to the blunted Asp718I site of pCGN5139 and the napin 3' was closest to the blunted HindIII site was subjected to sequence analysis to confirm both the insert orientation and the integrity of cloning junctions. The resulting plasmid was designated pCGN8622.

The plasmid pCGN8619 was constructed by ligating oligonucleotides 5'-TCGACCTGCAGGAAGCTTGC GGCCGCGGATCC -3' (SEQ ID NO:43) and 5'-TCGAGGATCCGCGGCCGCAAGCTTCCTGCAGG-3' (SEQ ID NO:44) into SalI/XhoI-digested pCGN7770. A fragment containing the napin promoter, polylinker and napin 3' region was removed from pCGN8619 by digestion with Asp718I; the fragment was blunt-ended by filling in the 5' overhangs with Klenow fragment then ligated into pCGN5139 that had been digested with Asp718I and HindIII and blunt-ended by filling in the 5' overhangs with Klenow fragment. A plasmid containing the insert oriented so that the napin promoter was closest to the blunted Asp718I site of pCGN5139 and the napin 3' was closest to the blunted HindIII site was subjected to sequence analysis to confirm both the insert orientation and the integrity of cloning junctions. The resulting plasmid was designated pCGN8623.

The plasmid pCGN8620 was constructed by ligating oligonucleotides 5'-TCGAGGATCCGCGGCCGCAAGCTTCCTGCAGGAGCT -3' (SEQ ID NO:45) and 5'-CCTGCAGGAAGCTTGC GGCCGCGGATCC-3' (SEQ ID NO:46) into SalI/SacI-digested pCGN7787. A fragment containing the d35S promoter, polylinker and tml 3' region was removed from pCGN8620 by complete digestion with Asp718I and partial digestion with NotI. The fragment was blunt-ended by filling in the 5' overhangs with Klenow fragment then ligated into pCGN5139 that had been digested with Asp718I and HindIII and blunt-ended by filling in the 5' overhangs with Klenow fragment. A plasmid containing the insert oriented so that the d35S promoter was closest to the blunted Asp718I site of pCGN5139 and the tml 3' was closest to the blunted HindIII site was subjected to sequence analysis to confirm both the insert orientation and the integrity of cloning junctions. The resulting plasmid was designated pCGN8624.

The plasmid pCGN8621 was constructed by ligating oligonucleotides 5'-TCGACCTGCAGGAAGCTTGC GGCCGCGGATCCAGCT -3' (SEQ ID NO:47) and 5'-GGATCCGCGGCCGCAAGCTTCCTGCAGG-3' (SEQ ID NO:48) into

SalI/SacI-digested pCGN7787. A fragment containing the d35S promoter, polylinker and tml 3' region was removed from pCGN8621 by complete digestion with Asp718I and partial digestion with NotI. The fragment was blunt-ended by filling in the 5' overhangs with Klenow fragment then ligated into pCGN5139 that had been digested with Asp718I and HindIII and blunt-ended by filling in the 5' overhangs with Klenow fragment. A plasmid containing the insert oriented so that the d35S promoter was closest to the blunted Asp718I site of pCGN5139 and the tml 3' was closest to the blunted HindIII site was subjected to sequence analysis to confirm both the insert orientation and the integrity of cloning junctions. The resulting plasmid was designated pCGN8625.

The plasmid construct pCGN8640 is a modification of pCGN8624 described above. A 938bp PstI fragment isolated from transposon Tn7 which encodes bacterial spectinomycin and streptomycin resistance (Fling et al. (1985), *Nucleic Acids Research* 13(19):7095-7106), a determinant for *E. coli* and *Agrobacterium* selection, was blunt ended with Pfu polymerase. The blunt ended fragment was ligated into pCGN8624 that had been digested with SpeI and blunt ended with Pfu polymerase. The region containing the PstI fragment was sequenced to confirm both the insert orientation and the integrity of cloning junctions.

The spectinomycin resistance marker was introduced into pCGN8622 and pCGN8623 as follows. A 7.7 Kbp AvrII-SnaBI fragment from pCGN8640 was ligated to a 10.9 Kbp AvrII-SnaBI fragment from pCGN8623 or pCGN8622, described above. The resulting plasmids were pCGN8641 and pCGN8643, respectively.

The plasmid pCGN8644 was constructed by ligating oligonucleotides 5'-GATCACCTGCAGGAAGCTTGCGGCCGCGGATCCAATGCA-3' (SEQ ID NO:49) and 5'-TTGGATCCGCGGCCGCAAGCTTCCTGCAGGT-3' (SEQ ID NO:50) into BamHI-PstI digested pCGN8640.

Synthetic oligonucleotides were designed for use in Polymerase Chain Reactions (PCR) to amplify the coding sequences of ATPT2, ATPT3, ATPT4, ATPT8, and ATPT12 for the preparation of expression constructs and are provided in Table 2 below.

5 **Table 2:**

Name	Restriction Site	Sequence	SEQ ID NO:
ATPT2	5' NotI	GGATCCGCGGCCGCACAATGGAGTC TCTGCTCTCTAGTTCT	51
ATPT2	3' SseI	GGATCCTGCAGGTCACCTTCAAAAAA GGTAACAGCAAGT	52
ATPT3	5' NotI	GGATCCGCGGCCGCACAATGGCGTT TTTGGGCTCTCCCGTGTTT	53
ATPT3	3' SseI	GGATCCTGCAGGTTATTGAAAACCTT CTTCCAAGTACAAC	54
ATPT4	5' NotI	GGATCCGCGGCCGCACAATGTGGCG AAGATCTGTTGTT	55
ATPT4	3' SseI	GGATCCTGCAGGTCATGGAGAGTAG AAGGAAGGAGCT	56
ATPT8	5' NotI	GGATCCGCGGCCGCACAATGGTACT TGCCGAGGTTCCAAAGCTTGCCTCT	57
ATPT8	3' SseI	GGATCCTGCAGGTCACCTGTTTCTGG TGATGACTCTAT	58
ATPT12	5' NotI	GGATCCGCGGCCGCACAATGACTTC GATTCTCAACACT	59
ATPT12	3' SseI	GGATCCTGCAGGTCAGTGTTGCGAT GCTAATGCCGT	60

The coding sequences of ATPT2, ATPT3, ATPT4, ATPT8, and ATPT12 were all amplified using the respective PCR primers shown in Table 2 above and cloned into the TopoTA vector (Invitrogen). Constructs containing the respective prenyltransferase
 10 sequences were digested with NotI and Sse8387I and cloned into the turbobinary vectors described above.

The sequence encoding ATPT2 prenyltransferase was cloned in the sense orientation into pCGN8640 to produce the plant transformation construct pCGN10800 (Figure 2). The ATPT2 sequence is under control of the 35S promoter.

The ATPT2 sequence was also cloned in the antisense orientation into the construct pCGN8641 to create pCGN10801 (Figure 3). This construct provides for the antisense expression of the ATPT2 sequence from the napin promoter.

The ATPT2 coding sequence was also cloned in the sense orientation into the
5 vector pCGN8643 to create the plant transformation construct pCGN10822

The ATPT2 coding sequence was also cloned in the antisense orientation into the vector pCGN8644 to create the plant transformation construct pCGN10803 (Figure 4).

The ATPT4 coding sequence was cloned into the vector pCGN864 to create the plant transformation construct pCGN10806 (Figure 5). The ATPT2 coding sequence was
10 cloned into the vector TopoTA™ vector from Invitrogen, to create the plant transformation construct pCGN10807 (Figure 6). The ATPT3 coding sequence was cloned into the TopoTA vector to create the plant transformation construct pCGN10808 (Figure 7). The ATPT3 coding sequence was cloned in the sense orientation into the vector pCGN8640 to create the plant transformation construct pCGN10809 (Figure 8). The
15 ATPT3 coding sequence was cloned in the antisense orientation into the vector pCGN8641 to create the plant transformation construct pCGN10810 (Figure 9). The ATPT3 coding sequence was cloned into the vector pCGN8643 to create the plant transformation construct pCGN10811 (Figure 10). The ATPT3 coding sequence was cloned into the vector pCGN8644 to create the plant transformation construct
20 pCGN10812 (Figure 11). The ATPT4 coding sequence was cloned into the vector pCGN8640 to create the plant transformation construct pCGN10813 (Figure 12). The ATPT4 coding sequence was cloned into the vector pCGN8641 to create the plant transformation construct pCGN10814 (Figure 13). The ATPT4 coding sequence was cloned into the vector pCGN8643 to create the plant transformation construct
25 pCGN10815 (Figure 14). The ATPT4 coding sequence was cloned in the antisense orientation into the vector pCGN8644 to create the plant transformation construct pCGN10816 (Figure 15). The ATPT8 coding sequence was cloned in the sense orientation into the vector pCGN8643 to create the plant transformation construct pCGN10819 (Figure 17). The ATPT12 coding sequence was cloned into the vector

pCGN8640 to create the plant transformation construct pCGN10824 (Figure 18). The ATPT12 coding sequence was cloned into the vector pCGN8643 to create the plant transformation construct pCGN10825 (Figure 19). The ATPT8 coding sequence was cloned into the vector pCGN8640 to create the plant transformation construct
5 pCGN10826 (Figure 20).

Example 3: Plant Transformation with Prenyl Transferase Constructs

Transgenic *Brassica* plants are obtained by *Agrobacterium*-mediated transformation as described by Radke *et al.* (*Theor. Appl. Genet.* (1988) 75:685-694;
10 *Plant Cell Reports* (1992) 11:499-505). Transgenic *Arabidopsis thaliana* plants may be obtained by *Agrobacterium*-mediated transformation as described by Valverkens *et al.*, (*Proc. Nat. Acad. Sci.* (1988) 85:5536-5540), or as described by Bent *et al.* ((1994), *Science* 265:1856-1860), or Bechtold *et al.* ((1993), *C.R.Acad.Sci, Life Sciences* 316:1194-1199). Other plant species may be similarly transformed using
15 related techniques.

Alternatively, microprojectile bombardment methods, such as described by Klein *et al.* (*Bio/Technology* 10:286-291) may also be used to obtain nuclear transformed plants.

20 Example 4: Identification of Additional Prenyltransferases

Additional BLAST searches were performed using the ATPT2 sequence, a sequence in the class of aromatic prenyltransferases. ESTs, and in some case, full-length coding regions, were identified in proprietary DNA libraries.

Soy full-length homologs to ATPT2 were identified by a combination of
25 BLAST (using ATPT2 protein sequence) and 5' RACE. Two homologs resulted (SEQ ID NO:95 and SEQ ID NO:96). Translated amino acid sequences are provided by SEQ ID NO:97 and SEQ ID NO:98.

A rice est ATPT2 homolog is shown in SEQ ID NO:99 (obtained from BLAST using the wheat ATPT2 homolog).

Other homolog sequences were obtained using ATPT2 and PSI-BLAST, including est sequences from wheat (SEQ ID NO:100), leek (SEQ ID NOs:101 and 102), canola (SEQ ID NO:103), corn (SEQ ID NOs:104, 105 and 106), cotton (SEQ ID NO:107) and tomato (SEQ ID NO:108).

5 A PSI-Blast profile generated using the *E. coli* ubiA (genbank accession 1790473) sequence was used to analyze the *Synechocystis* genome. This analysis identified 5 open reading frames (ORFs) in the *Synechocystis* genome that were potentially prenyltransferases; slr0926 (annotated as ubiA (4-hydroxybenzoate-octaprenyltransferase, SEQ ID NO:32), slr1899 (annotated as ctaB (cytochrome c
10 oxidase folding protein, SEQ ID NO:33), slr0056 (annotated as g4 (chlorophyll synthase 33 kd subunit, SEQ ID NO:34), slr1518 (annotated as menA (menaquinone biosynthesis protein, SEQ ID NO:35), and slr1736 (annotated as a hypothetical protein of unknown function (SEQ ID NO:36).

15 4A. *Synechocystis* Knock-outs

To determine the functionality of these ORFs and their involvement, if any, in the biosynthesis of tocopherols, knockouts constructs were made to disrupt the ORF identified in *Synechocystis*.

Synthetic oligos were designed to amplify regions from the 5' (5'-
20 TAATGTGTACATTGTCGGCCTC (17365') (SEQ ID NO:61) and 5'-
GCAATGTAACATCAGAGATTTTGAGACACAACGTGGCTTTCCACAATTCC
CCGCACCGTC (1736kanpr1)) (SEQ ID NO:62) and 3' (5'-
AGGCTAATAAGCACAAATGGGA (17363')) (SEQ ID NO:63) and 5'-
GGTATGAGTCAGCAACACCTTCTTCACGAGGCAGACCTCAGC
25 GGAATTGGTTTAGGTTATCCC (1736kanpr2)) (SEQ ID NO:64) ends of the
slr1736 ORF. The 1736kanpr1 and 1736kanpr2 oligos contained 20 bp of homology to the slr1736 ORF with an additional 40 bp of sequence homology to the ends of the kanamycin resistance cassette. Separate PCR steps were completed with these oligos and the products were gel purified and combined with the kanamycin resistance gene

from puc4K (Pharmacia) that had been digested with *HincII* and gel purified away from the vector backbone. The combined fragments were allowed to assemble without oligos under the following conditions: 94°C for 1 min, 55°C for 1 min, 72°C for 1 min plus 5 seconds per cycle for 40 cycles using pfu polymerase in 100ul reaction volume (Zhao, H and Arnold (1997) *Nucleic Acids Res.* 25(6):1307-1308). One microliter or five microliters of this assembly reaction was then amplified using 5' and 3' oligos nested within the ends of the ORF fragment, so that the resulting product contained 100-200 bp of the 5' end of the *Synechocystis* gene to be knocked out, the kanamycin resistance cassette, and 100-200 bp of the 3' end of the gene to be knocked out. This PCR product was then cloned into the vector pGemT easy (Promega) to create the construct pMON21681 and used for *Synechocystis* transformation.

Primers were also synthesized for the preparation of *Synechocystis* knockout constructs for the other sequences using the same method as described above, with the following primers. The *ubiA* 5' sequence was amplified using the primers 5'-GGATCCATGGTT GCCCAAACCCCATC (SEQ ID NO:65) and 5'-GCAATGTAACATCAGAGA TTTTGAGACACAACG TGGCTTTGGGTAAGCAACAATGACCGGC (SEQ ID NO:66). The 3' region was amplified using the synthetic oligonucleotide primers 5'-GAATTCTCAAAGCCAGCCCAGTAAC (SEQ ID NO:67) and 5'-GGTATGAGTC AGCAACACCTTCTTCACGAGGCAGACCTCAGCGGGTGCGAAAAGGGTTTT CCC (SEQ ID NO:68). The amplification products were combined with the kanamycin resistance gene from puc4K (Pharmacia) that had been digested with *HincII* and gel purified away from the vector backbone. The annealed fragment was amplified using 5' and 3' oligos nested within the ends of the ORF fragment (5'-CCAGTGGTTTAGGCTGTGTGGTC (SEQ ID NO:69) and 5'-CTGAGTTGGATGTATTGGATC (SEQ ID NO:70)), so that the resulting product contained 100-200 bp of the 5' end of the *Synechocystis* gene to be knocked out, the kanamycin resistance cassette, and 100-200 bp of the 3' end of the gene to be knocked

out. This PCR product was then cloned into the vector pGemT easy (Promega) to create the construct pMON21682 and used for *Synechocystis* transformation.

Primers were also synthesized for the preparation of *Synechocystis* knockout constructs for the other sequences using the same method as described above, with the following primers. The sl11899 5' sequence was amplified using the primers 5'-
5 GGATCCATGGTTACTT CGACAAAAATCC (SEQ ID NO:71) and 5'-
GCAATGTAACATCAGAG
ATTTTGAGACACAACGTGGCTTTGCTAGGCAACCGCTTAGTAC (SEQ ID
NO:72). The 3' region was amplified using the synthetic oligonucleotide primers 5'-
10 GAATTCTTAACCCAACAGTAAAGTTCCC (SEQ ID NO:73) and 5'-
GGTATGAGTCAGC
AACACCTTCTTCACGAGGCAGACCTCAGCGCCGGCATTGTCTTTTACATG
(SEQ ID NO:74). The amplification products were combined with the kanamycin
resistance gene from puc4K (Pharmacia) that had been digested with *HincII* and gel
15 purified away from the vector backbone. The annealed fragment was amplified using
5' and 3' oligos nested within the ends of the ORF fragment (5'-
GGAACCCTTGCAGCCGCTTC (SEQ ID NO:75)
and 5'- GTATGCCCAACTGGTGCAGAGG (SEQ ID NO:76)), so that the resulting
product contained 100-200 bp of the 5' end of the *Synechocystis* gene to be knocked
20 out, the kanamycin resistance cassette, and 100-200 bp of the 3' end of the gene to be
knocked out. This PCR product was then cloned into the vector pGemT easy
(Promega) to create the construct pMON21679 and used for *Synechocystis*
transformation.

Primers were also synthesized for the preparation of *Synechocystis* knockout
25 constructs for the other sequences using the same method as described above, with the
following primers. The slr0056 5' sequence was amplified using the primers 5'-
GGATCCATGTCTGACACACAAAATACCG (SEQ ID NO:77) and 5'-
GCAATGTAACATCAGAGATTTTGAGACACAACGTGGCTTTGCGCCAATACC
AGCCACCAACAG (SEQ ID NO:78). The 3' region was amplified using the

synthetic oligonucleotide primers 5'- GAATTCTCAAAT CCCCGCATGGCCTAG
(SEQ ID NO:79) and 5'-
GGTATGAGTCAGCAACACCTTCTTCACGAGGCAGACCTCAGCGGCCTACG
GCTTGGACGTGTGGG (SEQ ID NO:80). The amplification products were
5 combined with the kanamycin resistance gene from puc4K (Pharmacia) that had been
digested with *HincII* and gel purified away from the vector backbone. The annealed
fragment was amplified using 5' and 3' oligos nested within the ends of the ORF
fragment (5'- CACTTGGATTCCCCTGATCTG (SEQ ID NO:81) and 5'-
GCAATACCCGCTTGGAAAACG (SEQ ID NO:82)), so that the resulting product
10 contained 100-200 bp of the 5' end of the *Synechocystis* gene to be knocked out, the
kanamycin resistance cassette, and 100-200 bp of the 3' end of the gene to be knocked
out. This PCR product was then cloned into the vector pGemT easy (Promega) to
create the construct pMON21677 and used for *Synechocystis* transformation.

Primers were also synthesized for the preparation of *Synechocystis* knockout
15 constructs for the other sequences using the same method as described above, with the
following primers. The slr1518 5' sequence was amplified using the primers 5'-
GGATCCATGACCGAAT CTTCGCCCCTAGC (SEQ ID NO:83) and 5'-
GCAATGTAAACATCAGAGATTTTGA GACACAACGTGGC
TTTCAATCCTAGGTAGCCGAGGCG (SEQ ID NO:84). The 3' region was
20 amplified using the synthetic oligonucleotide primers 5'-
GAATTCTTAGCCCAGGCC AGCCCAGCC (SEQ ID NO:85) and 5'-
GGTATGAGTCAGCAACACCTTCTTCACGA
GGCAGACCTCAGCGGGGAATTGATTTGTTAATTACC (SEQ ID NO:86). The
amplification products were combined with the kanamycin resistance gene from
25 puc4K (Pharmacia) that had been digested with *HincII* and gel purified away from the
vector backbone. The annealed fragment was amplified using 5' and 3' oligos nested
within the ends of the ORF fragment (5'- GCGATCGCCATTATCGCTTGG (SEQ ID
NO:87) and 5'- GCAGACTGGCAATTATCAGTAACG (SEQ ID NO:88)), so that
the resulting product contained 100-200 bp of the 5' end of the *Synechocystis* gene to

be knocked out, the kanamycin resistance cassette, and 100-200 bp of the 3' end of the gene to be knocked out. This PCR product was then cloned into the vector pGemT easy (Promega) to create the construct pMON21680 and used for *Synechocystis* transformation.

5

4B. Transformation of *Synechocystis*

Cells of *Synechocystis* 6803 were grown to a density of approximately 2×10^8 cells per ml and harvested by centrifugation. The cell pellet was re-suspended in fresh BG-11 medium (ATCC Medium 616) at a density of 1×10^9 cells per ml and used immediately for transformation. One-hundred microliters of these cells were mixed with 5 μ l of mini prep DNA and incubated with light at 30C for 4 hours. This mixture was then plated onto nylon filters resting on BG-11 agar supplemented with TES pH8 and allowed to grow for 12-18 hours. The filters were then transferred to BG-11 agar + TES + 5ug/ml kanamycin and allowed to grow until colonies appeared within 7-10 days (Packer and Glazer, 1988). Colonies were then picked into BG-11 liquid media containing 5 μ g/ml kanamycin and allowed to grow for 5 days. These cells were then transferred to Bg-11 media containing 10ug/ml kanamycin and allowed to grow for 5 days and then transferred to Bg-11 + kanamycin at 25ug/ml and allowed to grow for 5 days. Cells were then harvested for PCR analysis to determine the presence of a disrupted ORF and also for HPLC analysis to determine if the disruption had any effect on tocopherol levels.

PCR analysis of the *Synechocystis* isolates for slr1736 and sll1899 showed complete segregation of the mutant genome, meaning no copies of the wild type genome could be detected in these strains. This suggests that function of the native gene is not essential for cell function. HPLC analysis of these same isolates showed that the sll1899 strain had no detectable reduction in tocopherol levels. However, the strain carrying the knockout for slr1736 produced no detectable levels of tocopherol.

The amino acid sequences for the *Synechocystis* knockouts are compared using ClustalW, and are provided in Table 3 below. Provided are the percent identities,

percent similarity, and the percent gap. The alignment of the sequences is provided in Figure 21.

Table 3:

	Slr1736	slr0926	sll1899	slr0056	slr1518
slr1736 %identity		14	12	18	11
%similar		29	30	34	26
%gap		8	7	10	5
slr0926 %identity			20	19	14
%similar			39	32	28
%gap			7	9	4
sll1899 %identity				17	13
%similar				29	29
%gap				12	9
slr0056 %identity					15
%similar					31
%gap					8
slr1518 %identity					
%similar					
%gap					

5

Amino acid sequence comparisons are performed using various *Arabidopsis* prenyltransferase sequences and the *Synechocystis* sequences. The comparisons are presented in Table 4 below. Provided are the percent identities, percent similarity, and the percent gap. The alignment of the sequences is provided in Figure 22.

4C. Phytyl Prenyltransferase Enzyme Assays

[³H] Homogentisic acid in 0.1% H₃PO₄ (specific radioactivity 40 Ci/mmol).

Phytyl pyrophosphate was synthesized as described by Joo, *et al.* (1973) *Can J.*

- 5 *Biochem.* 51:1527. 2-methyl-6-phytylquinol and 2,3-dimethyl-5-phytylquinol were synthesized as described by Soll, *et al.* (1980) *Phytochemistry* 19:215. Homogentisic acid, α, β, δ, and γ-tocopherol, and tocol, were purchased commercially.

- The wild-type strain of *Synechocystis* sp. PCC 6803 was grown in BG11 medium with bubbling air at 30°C under 50 μE.m⁻².s⁻¹ fluorescent light, and 70% relative
10 humidity. The growth medium of slr1736 knock-out (potential PPT) strain of this organism was supplemented with 25 μg mL⁻¹ kanamycin. Cells were collected from 0.25 to 1 liter culture by centrifugation at 5000 *g* for 10 min and stored at -80°C.

- Total membranes were isolated according to Zak's procedures with some modifications (Zak, *et al.* (1999) *Eur J. Biochem* 261:311). Cells were broken on a
15 French press. Before the French press treatment, the cells were incubated for 1 hour with lysozyme (0.5%, w/v) at 30 °C in a medium containing 7 mM EDTA, 5 mM NaCl and 10 mM Hepes-NaOH, pH 7.4. The spheroplasts were collected by centrifugation at 5000 *g* for 10 min and resuspended at 0.1 - 0.5 mg chlorophyll·mL⁻¹ in 20 mM potassium phosphate buffer, pH 7.8. Proper amount of protease inhibitor cocktail and
20 DNAase I from Boehringer Mannheim were added to the solution. French press treatments were performed two to three times at 100 MPa. After breakage, the cell suspension was centrifuged for 10 min at 5000*g* to pellet unbroken cells, and this was followed by centrifugation at 100 000 *g* for 1 hour to collect total membranes. The final pellet was resuspended in a buffer containing 50 mM Tris-HCL and 4 mM MgCl₂.

- 25 Chloroplast pellets were isolated from 250 g of spinach leaves obtained from local markets. Devined leaf sections were cut into grinding buffer (2 l /250 g leaves) containing 2 mM EDTA, 1 mM MgCl₂, 1 mM MnCl₂, 0.33 M sorbitol, 0.1% ascorbic acid, and 50 mM Hepes at pH 7.5. The leaves were homogenized for 3 sec three times in a 1-L blender, and filtered through 4 layers of miracloth. The supernatant was then

centrifuged at 5000g for 6 min. The chloroplast pellets were resuspended in small amount of grinding buffer (Douce, *et al* Methods in Chloroplast Molecular Biology, 239 (1982))

Chloroplasts in pellets can be broken in three ways. Chloroplast pellets were first aliquoted in 1 mg of chlorophyll per tube, centrifuged at 6000 rpm for 2 min in microcentrifuge, and grinding buffer was removed. Two hundred microliters of Triton X-100 buffer (0.1% Triton X-100, 50 mM Tris-HCl pH 7.6 and 4 mM MgCl₂) or swelling buffer (10 mM Tris pH 7.6 and 4 mM MgCl₂) was added to each tube and incubated for ½ hour at 4°C. Then the broken chloroplast pellets were used for the assay immediately. In addition, broken chloroplasts can also be obtained by freezing in liquid nitrogen and stored at -80°C for ½ hour, then used for the assay.

In some cases chloroplast pellets were further purified with 40%/ 80% percoll gradient to obtain intact chloroplasts. The intact chloroplasts were broken with swelling buffer, then either used for assay or further purified for envelope membranes with 20.5%/ 31.8% sucrose density gradient (Sol, *et al* (1980) *supra*). The membrane fractions were centrifuged at 100 000g for 40 min and resuspended in 50 mM Tris-HCl pH 7.6, 4 mM MgCl₂.

Various amounts of [³H]HGA, 40 to 60 µM unlabelled HGA with specific activity in the range of 0.16 to 4 Ci/mmol were mixed with a proper amount of 1M Tris-NaOH pH 10 to adjust pH to 7.6. HGA was reduced for 4 min with a trace amount of solid NaBH₄. In addition to HGA, standard incubation mixture (final vol 1 mL) contained 50 mM Tris-HCl, pH 7.6, 3-5 mM MgCl₂, and 100 µM phytyl pyrophosphate. The reaction was initiated by addition of *Synechocystis* total membranes, spinach chloroplast pellets, spinach broken chloroplasts, or spinach envelope membranes. The enzyme reaction was carried out for 2 hour at 23°C or 30°C in the dark or light. The reaction is stopped by freezing with liquid nitrogen, and stored at -80°C or directly by extraction.

A constant amount of tocol was added to each assay mixture and reaction products were extracted with a 2 mL mixture of chloroform/methanol (1:2, v/v) to give a

monophasic solution. NaCl solution (2 mL; 0.9%) was added with vigorous shaking. This extraction procedure was repeated three times. The organic layer containing the prenylquinones was filtered through a 20 µm filter, evaporated under N₂ and then resuspended in 100 µL of ethanol.

- 5 The samples were mainly analyzed by Normal-Phase HPLC method (Isocratic 90% Hexane and 10% Methyl-t-butyl ether), and use a Zorbax silica column, 4.6 x 250 mm. The samples were also analyzed by Reversed-Phase HPLC method (Isocratic 0.1% H₃PO₄ in MeOH), and use a Vydac 201HS54 C18 column; 4.6 x 250 mm coupled with an All-tech C18 guard column. The amount of products were calculated
10 based on the substrate specific radioactivity, and adjusted according to the % recovery based on the amount of internal standard.

The amount of chlorophyll was determined as described in Arnon (1949) *Plant Physiol.* 24:1. Amount of protein was determined by the Bradford method using gamma globulin as a standard (Bradford, (1976) *Anal. Biochem.* 72:248)

- 15 Results of the assay demonstrate that 2-Methyl-6-Phytylplastoquinone is not produced in the *Synechocystis* slr1736 knockout preparations. The results of the phytol prenyltransferase enzyme activity assay for the slr1736 knock out are presented in Figure 23.

20 4D. Complementation of the slr1736 knockout with ATPT2

- In order to determine whether ATPT2 could complement the knockout of slr1736 in *Synechocystis* 6803, a plasmid was constructed to express the ATPT2 sequence from the TAC promoter. A vector, plasmid psl1211, was obtained from the lab of Dr. Himadri Pakrasi of Washington University, and is based on the plasmid
25 RSF1010 which is a broad host range plasmid (Ng W.-O., Zentella R., Wang, Y., Taylor J-S. A., Pakrasi, H.B. 2000. *phrA*, the major photoreactivating factor in the cyanobacterium *Synechocystis* sp. strain PCC 6803 codes for a cyclobutane pyrimidine dimer specific DNA photolyase. *Arch. Microbiol.* (in press)). The ATPT2 gene was isolated from the vector pCGN10817 by PCR using the following primers.

ATPT2nco.pr 5'-CCATGGATTCGAGTAAAGTTGTCGC (SEQ ID NO:89);
ATPT2ri.pr- 5'-GAATTCACCTTCAAAAAAGGTAACAG (SEQ ID NO:90). These
primers will remove approximately 112 BP from the 5' end of the ATPT2 sequence,
which is thought to be the chloroplast transit peptide. These primers will also add an
5 NcoI site at the 5' end and an EcoRI site at the 3' end which can be used for sub-
cloning into subsequent vectors. The PCR product from using these primers and
pCGN10817 was ligated into pGEM T easy and the resulting vector pMON21689 was
confirmed by sequencing using the m13forward and m13reverse primers. The
NcoI/EcoRI fragment from pMON21689 was then ligated with the EagI/EcoRI and
10 EagI/NcoI fragments from psl1211 resulting in pMON21690. The plasmid
pMON21690 was introduced into the slr1736 *Synechocystis* 6803 KO strain via
conjugation. Cells of sl906 (a helper strain) and DH10B cells containing
pMON21690 were grown to log phase (O.D. 600= 0.4) and 1 ml was harvested by
centrifugation. The cell pellets were washed twice with a sterile BG-11 solution and
15 resuspended in 200 ul of BG-11. The following was mixed in a sterile eppendorf
tube: 50 ul SL906, 50 ul DH10B cells containing pMON21690, and 100 ul of a fresh
culture of the slr1736 *Synechocystis* 6803 KO strain (O.D. 730 = 0.2-0.4). The cell
mixture was immediately transferred to a nitrocellulose filter resting on BG-11 and
incubated for 24 hours at 30C and 2500 LUX(50 ue) of light. The filter was then
20 transferred to BG-11 supplemented with 10ug/ml Gentamycin and incubated as above
for ~5 days. When colonies appeared, they were picked and grown up in liquid BG-
11 + Gentamycin 10 ug/ml. (Elhai, J. and Wolk, P. 1988. Conjugal transfer of DNA
to Cyanobacteria. *Methods in Enzymology* 167, 747-54) The liquid cultures were then
assayed for tocopherols by harvesting 1ml of culture by centrifugation, extracting with
25 ethanol/pyrogallol, and HPLC separation. The slr1736 *Synechocystis* 6803 KO strain,
did not contain any detectable tocopherols, while the slr1736 *Synechocystis* 6803 KO
strain transformed with pmon21690 contained detectable alpha tocopherol. A
Synechocystis 6803 strain transformed with psl1211(vector control) produced alpha
tocopherol as well.

4E: Additional Evidence of Prenyltransferase Activity

To test the hypothesis that slr1736 or ATPT2 are sufficient as single genes to obtain phytol prenyltransferase activity, both genes were expressed in SF9 cells and in yeast. When either slr1736 or ATPT2 were expressed in insect cells (Table 5) or in yeast, phytol prenyltransferase activity was detectable in membrane preparations, whereas membrane preparations of the yeast vector control, or membrane preparations of insect cells did not exhibit phytol prenyltransferase activity.

10 **Table 5:** Phytol prenyltransferase activity

Enzyme source	Enzyme activity [pmol/mg x h]
slr1736 expressed in SF9 cells	20
ATPT2 expressed in SF9 cells	6
SF9 cell control	< 0.05
<i>Synechocystis</i> 6803	0.25
Spinach chloroplasts	0.20

Example 5: Transgenic Plant Analysis

15 5A. *Arabidopsis*

Arabidopsis plants transformed with constructs for the sense or antisense expression of the ATPT proteins were analyzed by High Pressure Liquid Chromatography (HPLC) for altered levels of total tocopherols, as well as altered levels of specific tocopherols (alpha, beta, gamma, and delta tocopherol).

20 Extracts of leaves and seeds were prepared for HPLC as follows. For seed extracts, 10 mg of seed was added to 1 g of microbeads (Biospec) in a sterile microfuge tube to which 500 ul 1% pyrogallol (Sigma Chem)/ethanol was added. The mixture was shaken for 3 minutes in a mini Beadbeater (Biospec) on "fast" speed.

The extract was filtered through a 0.2 μ m filter into an autosampler tube. The filtered extracts were then used in HPLC analysis described below.

Leaf extracts were prepared by mixing 30-50 mg of leaf tissue with 1 g microbeads and freezing in liquid nitrogen until extraction. For extraction, 500 μ l 1% pyrogallol in ethanol was added to the leaf/bead mixture and shaken for 1 minute on a
5 Beadbeater (Biospec) on "fast" speed. The resulting mixture was centrifuged for 4 minutes at 14,000 rpm and filtered as described above prior to HPLC analysis.

HPLC was performed on a Zorbax silica HPLC column (4.6 mm X 250 mm) with a fluorescent detection, an excitation at 290 nm, an emission at 336 nm, and
10 bandpass and slits. Solvent A was hexane and solvent B was methyl-t-butyl ether. The injection volume was 20 μ l, the flow rate was 1.5 ml/min, the run time was 12 min (40°C) using the gradient (Table 6):

Table 6:

15	<u>Time</u>	<u>Solvent A</u>	<u>Solvent B</u>
	0 min.	90%	10%
	10 min.	90%	10%
	11 min.	25%	75%
	12 min.	90%	10%

20

Tocopherol standards in 1% pyrogallol/ ethanol were also run for comparison (alpha tocopherol, gamma tocopherol, beta tocopherol, delta tocopherol, and tocopherol (tocol) (all from Matreya).

Standard curves for alpha, beta, delta, and gamma tocopherol were calculated
25 using Chemstation software. The absolute amount of component x is: Absolute amount of x = $\text{Response}_x \times \text{RF}_x \times \text{dilution factor}$ where Response_x is the area of peak x, RF_x is the response factor for component x ($\text{Amount}_x / \text{Response}_x$) and the dilution factor is 500 μ l. The ng/mg tissue is found by: total ng component/mg plant tissue.

Results of the HPLC analysis of seed extracts of transgenic *Arabidopsis* lines containing pMON10822 for the expression of ATPT2 from the napin promoter are provided in Figure 24.

HPLC analysis results of segregating T2 *Arabidopsis* seed tissue expressing
5 the ATPT2 sequence from the napin promoter (pCGN10822) demonstrates an increased level of tocopherols in the seed. Total tocopherol levels are increased as much as 50% over the total tocopherol levels of non-transformed (wild-type) *Arabidopsis* plants (Figure 25). Homozygous progeny from the top 3 lines (T3 seed) have up to a two-fold (100%) increase in total tocopherol levels over control
10 *Arabidopsis* seed (Figure 26.)

Furthermore, increases of particular tocopherols are also increased in transgenic *Arabidopsis* plants expressing the ATPT2 nucleic acid sequence from the napin promoter. Levels of delta tocopherol in these lines are increased greater than 3 fold over the delta tocopherol levels obtained from the seeds of wild type *Arabidopsis*
15 lines. Levels of gamma tocopherol in transgenic *Arabidopsis* lines expressing the ATPT2 nucleic acid sequence are increased as much as about 60% over the levels obtained in the seeds of non-transgenic control lines. Furthermore, levels of alpha tocopherol are increased as much as 3 fold over those obtained from non-transgenic control lines.

20 Results of the HPLC analysis of seed extracts of transgenic *Arabidopsis* lines containing pCGN10803 for the expression of ATPT2 from the enhanced 35S promoter (antisense orientation) are provided in Figure 25. Two lines were identified that have reduced total tocopherols, up to a ten-fold decrease observed in T3 seed compared to control *Arabidopsis* (Figure 27.)

25

5B. Canola

Brassica napus, variety SP30021, was transformed with pCGN10822 (napin-ATPT2-napin 3', sense orientation) using *Agrobacterium tumefaciens*-mediated

transformation. Flowers of the R0 plants were tagged upon pollination and developing seed was collected at 35 and 45 days after pollination (DAP).

Developing seed was assayed for tocopherol levels, as described above for *Arabidopsis*. Line 10822-1 shows a 20% increase of total tocopherols, compared to the wild-type control, at 45 DAP. Figure 28 shows total tocopherol levels measured in developing canola seed.

Example 6: Sequences to Tocopherol Cyclase

6A. Preparation of the slr1737 Knockout

10 The *Synechocystis* sp. 6803 slr1737 knockout was constructed by the following method. The GPS™-1 Genome Priming System (New England Biolabs) was used to insert, by a Tn7 Transposase system, a Kanamycin resistance cassette into *slr1737*. A plasmid from a *Synechocystis* genomic library clone containing 652 base pairs of the targeted orf (*Synechocystis* genome base pairs 1324051 – 1324703; 15 the predicted orf base pairs 1323672 – 1324763, as annotated by Cyanobase) was used as target DNA. The reaction was performed according to the manufacturers protocol. The reaction mixture was then transformed into *E. coli* DH10B electrocompetant cells and plated. Colonies from this transformation were then screened for transposon insertions into the target sequence by amplifying with M13 Forward and Reverse 20 Universal primers, yielding a product of 652 base pairs plus ~1700 base pairs, the size of the transposon kanamycin cassette, for a total fragment size of ~2300 base pairs. After this determination, it was then necessary to determine the approximate location of the insertion within the targeted orf, as 100 base pairs of orf sequence was estimated as necessary for efficient homologous recombination in *Synechocystis*. This 25 was accomplished through amplification reactions using either of the primers to the ends of the transposon, Primer S (5' end) or N (3' end), in combination with either a M13 Forward or Reverse primer. That is, four different primer combinations were used to map each potential knockout construct: Primer S – M13 Forward, Primer S – M13 Reverse, Primer N – M13 Forward, Primer N – M13 Reverse. The construct

used to transform *Synechocystis* and knockout slr1737 was determined to consist of a approximately 150 base pairs of slr1737 sequence on the 5' side of the transposon insertion and approximately 500 base pairs on the 3' side, with the transcription of the orf and kanamycin cassette in the same direction. The nucleic acid sequence of
5 slr1737 is provided in SEQ ID NO:38 the deduced amino acid sequence is provided in SEQ ID NO:39.

Cells of *Synechocystis* 6803 were grown to a density of $\sim 2 \times 10^8$ cells per ml and harvested by centrifugation. The cell pellet was re-suspended in fresh BG-11 medium at a density of 1×10^9 cells per ml and used immediately for transformation.
10 100 ul of these cells were mixed with 5 ul of mini prep DNA and incubated with light at 30C for 4 hours. This mixture was then plated onto nylon filters resting on BG-11 agar supplemented with TES pH8 and allowed to grow for 12-18 hours. The filters were then transferred to BG-11 agar + TES + 5ug/ml kanamycin and allowed to grow until colonies appeared within 7-10 days (Packer and Glazer, 1988). Colonies were
15 then picked into BG-11 liquid media containing 5 ug/ml kanamycin and allowed to grow for 5 days. These cells were then transferred to Bg-11 media containing 10ug/ml kanamycin and allowed to grow for 5 days and then transferred to Bg-11 + kanamycin at 25ug/ml and allowed to grow for 5 days. Cells were then harvested for PCR analysis to determine the presence of a disrupted ORF and also for HPLC
20 analysis to determine if the disruption had any effect on tocopherol levels.

PCR analysis of the *Synechocystis* isolates, using primers to the ends of the *slr1737* orf, showed complete segregation of the mutant genome, meaning no copies of the wild type genome could be detected in these strains. This suggests that function of the native gene is not essential for cell function. HPLC analysis of the
25 strain carrying the knockout for *slr1737* produced no detectable levels of tocopherol.

6B. The relation of slr1737 and slr1736

The slr1737 gene occurs in *Synechocystis* downstream and in the same orientation as slr1736, the phytyl prenyltransferase. In bacteria this proximity often

indicates an operon structure and therefore an expression pattern that is linked in all genes belonging to this operon. Occasionally such operons contain several genes that are required to constitute one enzyme. To confirm that slr1737 is not required for phytyl prenyltransferase activity, phytyl prenyltransferase was measured in extracts
5 from the *Synechocystis* slr1737 knockout mutant. Figure 29 shows that extracts from the *Synechocystis* slr1737 knockout mutant still contain phytyl prenyltransferase activity. The molecular organization of genes in *Synechocystis* 6803 is shown in A. Figures B and C show HPLC traces (normal phase HPLC) of reaction products obtained with membrane preparations from *Synechocystis* wild type and slr1737
10 membrane preparations, respectively.

The fact that slr1737 is not required for the PPT activity provides additional data that ATPT2 and slr1736 encode phytyl prenyltransferases.

6C *Synechocystis* Knockouts

15 *Synechocystis* 6803 wild type and *Synechocystis* slr1737 knockout mutant were grown photoautotrophically. Cells from a 20 ml culture of the late logarithmic growth phase were harvested and extracted with ethanol. Extracts were separated by isocratic normal-phase HPLC using a Hexane/Methyl-t-butyl ether (95/5) and a Zorbax silica column, 4.6 x 250 mm. Tocopherols and tocopherol intermediates were
20 detected by fluorescence (excitement 290 nm, emission 336 nm) (Figure 30).

Extracts of *Synechocystis* 6803 contained a clear signal of alpha-tocopherol. 2,3-Dimethyl-5-phytylplastoquinol was below the limit of detection in extracts from the *Synechocystis* wild type (C). In contrast, extracts from the *Synechocystis* slr1737 knockout mutant did not contain alpha-tocopherol, but contained 2,3-dimethyl-5-
25 phytylplastoquinol (D), indicating that the interruption of slr1737 has resulted in a block of the 2,3-dimethyl-5-phytylplastoquinol cyclase reaction.

Chromatograms of standard compounds alpha, beta, gamma, delta-tocopherol and 2,3-dimethyl-5-phytylplastoquinol are shown in A and B. Chromatograms of extracts from *Synechocystis* wild type and the *Synechocystis* slr1737 knockout mutant

are shown in C and D, respectively. Abbreviations: 2,3-DMPQ, 2,3-dimethyl-5-phytylplastoquinol.

6D. Incubation with Lysozyme treated *Synechocystis*

5 *Synechocystis* 6803 wild type and slr1737 knockout mutant cells from the late logarithmic growth phase (approximately 1g wet cells per experiment in a total volume of 3 ml) were treated with Lysozyme and subsequently incubated with S-adenosylmethionine, and phytylpyrophosphate, plus radiolabelled homogentisic acid. After 17h incubation in the dark at room temperature the samples were extracted
10 with 6 ml chloroform / methanol (1/2 v/v). Phase separation was obtained by the addition of 6 ml 0.9% NaCl solution. This procedure was repeated three times. Under these conditions 2,3-dimethyl-5-phytylplastoquinol is oxidized to form 2,3-dimethyl-5-phytylplastoquinone.

The extracts were analyzed by normal phase and reverse phase HPLC. Using
15 extracts from wild type *Synechocystis* cells radiolabelled gamma-tocopherol and traces of radiolabelled 2,3-dimethyl-5-phytylplastoquinone were detected. When extracts from the slr1737 knockout mutant were analyzed, only radiolabelled 2,3-dimethyl-5-phytylplastoquinone was detectable. The amount of 2,3-dimethyl-5-phytylplastoquinone was significantly increased compared to wild type extracts. Heat
20 treated samples of the wild type and the slr1737 knockout mutant did not produce radiolabelled 2,3-dimethyl-5-phytylplastoquinone, nor radiolabelled tocopherols. These results further support the role of the slr1737 expression product in the cyclization of 2,3-dimethyl-5-phytylplastoquinol.

25 6E. *Arabidopsis* Homologue to slr1737

An *Arabidopsis* homologue to slr1737 was identified from a BLASTALL search using *Synechocystis* sp 6803 gene slr1737 as the query, in both public and proprietary databases. SEQ ID NO:109 and SEQ ID NO:110 are the DNA and

translated amino acid sequences, respectively, of the *Arabidopsis* homologue to slr1737. The start is found at the ATG at base 56 in SEQ ID NO:109.

The sequences obtained for the homologue from the proprietary database differs from the public database (F4D11.30, BAC AL022537), in having a start site
5 471 base pairs upstream of the start identified in the public sequence. A comparison of the public and proprietary sequences is provided in Figure 31. The correct start correlates within the public database sequence is at 12080, while the public sequence start is given as being at 11609.

Attempts to amplify a slr1737 homologue were unsuccessful using primers
10 designed from the public database, while amplification of the gene was accomplished with primers obtained from SEQ ID NO:109.

Analysis of the protein sequence to identify transit peptide sequence predicted two potential cleavage sites, one between amino acids 48 and 49, and the other
15 between amino acids 98 and 99.

6F. slr1737 Protein Information

The slr1737 orf comprises 363 amino acid residues and has a predicted MW of 41kDa (SEQ ID NO: 39). Hydropathic analysis indicates the protein is hydrophilic (Figure 32).

20 The *Arabidopsis* homologue to slr1737 (SEQ ID xx) comprises 488 amino acid residues, has a predicted MW of 55kDa, and has a putative transit peptide sequence comprising the first 98 amino acids. The predicted MW of the mature form of the *Arabidopsis* homologue is 44kDa. The hydropathic plot for the *Arabidopsis* homologue also reveals that it is hydrophilic (Figure 33). Further blast analysis of
25 the *Arabidopsis* homologue reveals limited sequence identity (25 % sequence identity) with the beta-subunit of respiratory nitrate reductase. Based on the sequence identity to nitrate reductase, it suggests the slr1737 orf is an enzyme that likely involves general acid catalysis mechanism.

Investigation of known enzymes involved in tocopherol metabolism indicated that the best candidate corresponding to the general acid mechanism is the tocopherol cyclase. There are many known examples of cyclases including, tocopherol cyclase, chalcone isomerase, lycopene cyclase, and aristolochene synthase. By further
5 examination of the microscopic catalytic mechanism of phytoplastoquinol cyclization, as an example, chalcone isomerase has a catalytic mechanism most similar to tocopherol cyclase. (Figure 34).

Multiple sequence alignment was performed between slr1737, slr1737 *Arabidopsis* homologue and the *Arabidopsis* chalcone isomerase (Genbank:P41088)
10 (Figure 35). 65% of the conserved residues among the three enzymes are strictly conserved within the known chalcone isomerases. The crystal structure of alfalfa chalcone isomerase has been solved (Jez, Joseph M., Bowman, Marianne E., Dixon, Richard A., and Noel, Joseph P. (2000) "Structure and mechanism of the evolutionarily unique plant enzyme chalcone isomerase". *Nature Structural Biology*
15 7: 786-791.) It has been demonstrated tyrosine (Y) 106 of the alfalfa chalcone isomerase serves as the general acid during cyclization reaction (Genbank: P28012). The equivalent residue in slr1737 and the slr1737 *Arabidopsis* homolog is lysine (K), which is an excellent catalytic residue as general acid.

The information available from partial purification of tocopherol cyclase from
20 *Chlorella protothecoides* (U.S. Patent No. 5,432,069), i.e., described as being glycine rich, water soluble and with a predicted MW of 48-50kDa, is consistent with the protein informatics information obtained for the slr1737 and the *Arabidopsis* slr1737 homologue.

All publications and patent applications mentioned in this specification are
25 indicative of the level of skill of those skilled in the art to which this invention pertains. All publications and patent applications are herein incorporated by reference to the same extent as if each individual publication or patent application was specifically and individually indicated to be incorporated by reference.

Although the foregoing invention has been described in some detail by way of illustration and example for purposes of clarity of understanding, it will be obvious that certain changes and modifications may be practiced within the scope of the appended claim.

CLAIMS

What is claimed is:

1. An isolated nucleic acid sequence encoding a tocopherol cyclase.
- 5 2. An isolated nucleic acid sequence according to Claim 1, wherein said tocopherol cyclase is active in the cyclization of 2,3-dimethyl-5-phytylplastoquinol to tocopherol.
3. An isolated nucleic acid sequence according to Claim 1, wherein said tocopherol cyclase is active in the cyclization of 2,3-dimethyl-5-geranylgeranylplastoquinol to tocotrienol.
- 10 4. An isolated DNA sequence according to Claim 1, wherein said nucleic acid sequence is isolated from a eukaryotic cell source.
5. An isolated DNA sequence according to Claim 4, wherein said eukaryotic cell source is selected from the group consisting of mammalian, nematode, fungal, and plant cells.
6. The DNA encoding sequence of Claim 5 wherein said tocopherol cyclase protein is from
15 *Arabidopsis*.
7. The DNA encoding sequence of Claim 6 wherein said tocopherol cyclase protein is encoded by a sequence of SEQ ID NO:109.
8. The DNA encoding sequence of Claim 7 wherein said tocopherol cyclase protein has an amino acid sequence of SEQ ID NO:110.
- 20 9. The DNA encoding sequence of Claim 4 wherein said tocopherol cyclase protein is from a source selected from the group consisting of *Arabidopsis*, soybean, corn, rice, wheat, leek canola, , leek, cotton, and tomato.
10. An isolated DNA sequence according to Claim 4, wherein said prokaryotic source is a *Synechocystis* sp.
- 25 11. The DNA encoding sequence of Claim 10 wherein said tocopherol cyclase protein is encoded by a sequence of SEQ ID NO:38.
12. The DNA encoding sequence of Claim 10 wherein said tocopherol cyclase protein has an amino acid sequence of SEQ ID NO:39.

13. A nucleic acid construct comprising as operably linked components, a transcriptional initiation region functional in a host cell, a nucleic acid sequence encoding a tocopherol cyclase, and a transcriptional termination region.
14. A nucleic acid construct according to Claim 13, wherein said nucleic acid sequence
5 encoding tocopherol cyclase is obtained from an organism selected from the group consisting of a eukaryotic organism and a prokaryotic organism.
15. A nucleic acid construct according to Claim 14, wherein said nucleic acid sequence encoding tocopherol cyclase is obtained from a plant source.
16. A nucleic acid construct according to Claim 15, wherein said nucleic acid sequence
10 encoding tocopherol cyclase is obtained from a source selected from the group consisting of *Arabidopsis*, soybean, corn, rice, wheat, leek canola, , leek, cotton, and tomato.
17. A nucleic acid construct according to Claim 13, wherein said nucleic acid sequence encoding tocopherol cyclase is obtained from a *Synechocystis* sp.
18. A plant cell comprising the construct of 13.
- 15 19. A plant comprising a cell of Claim 18.
- 20 A feed composition produced from a plant according to Claim 19.
21. A seed comprising a cell of Claim 18.
- 22 Oil obtained from a seed of Claim 21.
23. A natural tocopherol rich refined and deodorised oil which has been produced by
20 a method of treating an oil according to Claim 22 by distilling under low pressure and high temperature, wherein said refined oil has reduced free fatty acids and a substantial percentage of tocopherol present in the pretreated oil.
24. A refined oil according to claim 23, wherein the pretreated oil is crude or pre-treated soybean oil.
- 25 25. A refined oil according to claim 23, wherein the refined oil is degummed and bleached.
26. A method for the alteration of the isoprenoid content in a host cell, said method comprising; transforming said host cell with a construct comprising as operably linked

components, a transcriptional initiation region functional in a host cell, a nucleic acid sequence encoding tocopherol cyclase, and a transcriptional termination region, wherein said isoprenoid compound selected from the group of tocopherols and tocotrienols .

27. The method according to Claim 26, wherein said host cell is selected from the group
5 consisting of a prokaryotic cell and a eukaryotic cell.

28. The method according to Claim 27, wherein said prokaryotic cell is a *Synechocystis* sp.

29. The method according to Claim 27, wherein said eukaryotic cell is a plant cell.

30. The method according to Claim 29, wherein said plant cell is obtained from a plant
selected from the group consisting of *Arabidopsis*, soybean, corn, rice, wheat, leek canola, ,
10 leek, cotton, and tomato.

31. A method for producing an isoprenoid compound of interest in a host cell, said method
comprising obtaining a transformed host cell, said host cell having and expressing in its
genome:

a construct having a DNA sequence encoding a tocopherol cyclase operably linked to a
15 transcriptional initiation region functional in a host cell,
wherein said isoprenoid compound selected from the group of tocopherols and tocotrienols.

32. The method according to Claim 31, wherein said host cell is selected from the group
consisting of a prokaryotic cell and a eukaryotic cell.

33. The method according to Claim 32, wherein said prokaryotic cell is a *Synechocystis* sp.

20 34. The method according to Claim 32, wherein said eukaryotic cell is a plant cell.

35. The method according to Claim 34, wherein said plant cell is obtained from a plant
selected from the group consisting wherein said compound selected from the group of
Arabidopsis, soybean, corn, rice, wheat, leek canola, , leek, cotton, and tomato.

36. A method for increasing the biosynthetic flux in a host cell toward production of
25 an isoprenoid compound, said method comprising; transforming said host cell with a
construct comprising as operably linked components, a transcriptional initiation
region functional in a host cell, a DNA encoding a tocopherol cyclase, and a
transcriptional termination region, wherein said isoprenoid compound selected from
the group of tocopherols and tocotrienols,.

37. The method according to Claim 36, wherein said host cell is selected from the group consisting of a prokaryotic cell and a eukaryotic cell.
38. The method according to Claim 37, wherein said prokaryotic cell is a *Synechocystis* sp.
39. The method according to Claim 37, wherein said eukaryotic cell is a plant cell.
- 5 40. The method according to Claim 39, wherein said plant cell is obtained from a plant selected from the group consisting *Arabidopsis*, soybean, corn, rice, wheat, leek canola, , leek, cotton, and tomato.
41. The method according to Claim 39, wherein said transcriptional initiation region is a seed-specific promoter.

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ATPT2: ---MDS---PSSSI---AAGG---FCKKON---LKLHSI---IVLRCD---SKVAKP---NR---NNLRP---DQGG : 59
 ATPT3: MAFFLSRVRRRLKGVVPS---SALLQSOHQELSN---PVTHYTNPKCYDEWNDYQVMSKQ---ELHOE---FVGWKNYRLICGMSS : 90
 ATPT4: ---MRRS---VVRFFSR---VSSLPNRLIPREL---CAVNSPPVSTKAGITGV---ASD---ANRFA---HSA : 69
 ATPT8: ---WLAEP---KLA---AAEFK---GVQGF---LLELMATA---LN---VRPDE---ALIG : 48
 ATPT12: ---MTS---HNVGT---HSRVTSVDRGVGLR---SDSVERRR---SGFLLIYESP---RNV---VPAAE---DID : 64
 S 6
 ATPT2 : SSLLLY---KHKSFR---TAGQPEAFDSNSKQSFDRSD---AFRFSR---HTV---GTVLVSILSV---LAVERKSDISPLFTGILEA : 141
 ATPT3 : SVLEGR---KDDKEKSDGVVKKASW---DLYLPPEVRGYAKLAR---KPKGTWLLAW---CYSMAL---AADPSLPSF---K---YMALFGG : 171
 ATPT4 : AATATAT---TG-EISSV---ANLAGE---GHYARCWELSK---AKLSMLVATS---GTG---GT---GNAL---PPL---C---YTCA : 137
 ATPT8 : ESTDIVT---SELVRQ---GIEITEMIHVASILLDDVL---DDETRVRGS---LNV---GNKMSVLADDELLS---RACG : 117
 ATPT12 : KVKASQTS---DKAPAGSSINQLLEK---ASQETNKWIRLOITKPM---TWP---ELV---GVCGAAAS---NHWTPED---VAKSILCM : 140
 I 1
 ATPT2 : VMAALMNIYIVGNOISSEVETKVNKP---YLPDASGEYSVNTGTALVASFQMS---FWG---GSGMPLFWA---LFSPL---GTAGINL : 224
 ATPT3 : AL---DARGAGC---INDIL---DOD---TKVDRTKIE---JASGL---EPFG---CEGQOLIA---LGILLQNNIS---RGLGESSLLLA : 248
 ATPT4 : GT---MHAASANS---NOQFENSNSKMKRTMLE---EPSCHE---SVPHAVATVAGASACLI---ASKTNMLAAG---LASANVLYIAP : 219
 ATPT8 : AL---AALKNTEVVALLATA---EHLVTGET---MENTSSTEORYSNDY---KQTYTKI---ASL---SCK---ANAV---GQTAEVAV : 190
 ATPT12 : MSGPQLTCYTO---INDYERD---DAINER---YRELPSCATSEPEVITQWMLLGG---LGAC---VNVAGCHTTPVTVILALGGLLS : 227
 n 6
 ATPT2 : ELNWRFRALVAMCINAVRAIE---QIAFVYLHIQTH---FGRPILFTRPI---FAPAFMSFFS---VVR---FKDIPD---D : 299
 ATPT3 : P---MKFTFPQ---FQGT---INWGAL---T---AKSG---APSI---VTP---LYLSG---CWTEVITD---YAHQKED---D---VK : 314
 ATPT4 : LKQLHPINTWGVV---GAPPLLEWA---AASQISYNSM---PAALYFQW---PHEFAVAFHCRNDYAAAGYKMLSLFPSPGKR : 300
 ATPT8 : LAFEYGRALGJAPQI---DDADFTITS---ASLEKGLSD---RHGVIA---PILFAMEEFPQREVVDO---D---NN : 259
 ATPT12 : PPKL---KQNGVGNFA---G---ASVSEPTWAGQ---APFS---KPPDVW---LILYS---IAG---LCA---VNDPKS---D : 294
 a 6
 ATPT2 : FCHRS---FSVTL---Q---KRVFTG---VTLQVYVYVILVNTSPFTSK---NISVGHVLA---TLWARAKSVLSKTEITS : 375
 ATPT3 : VGVKS---TALRQD---NKKL---TGTGFG---ASIGFT---ASGSADLWQIYAS---PAA---GQ---QIGTADLSSG---CSRKFVSNKW : 392
 ATPT4 : VAKNCFYMIPL---LAYDWGL---SSPFLS---LTL---TATASVYRDRTHKARKMFTES---FLPM---SGLLLRVSN---QOOLVEEAGL : 390
 ATPT8 : VDIAL---EYLKSK---GQO---RARE---MHEFNLA---AIGSPET---DNEDV---KRSRRALIDLTHRVITRK--- : 321
 ATPT12 : LGLQJ---LPVAGST---EPAV---VGA---DITOL---SVCGLISGKDYALA---L---L---PQ---QFKYFLKDPVKY---VKYQASAO : 373
 w 6
 ATPT2 : ---MFKLKYAE---YLLLPFLK--- : 393
 ATPT3 : GAIHPSGVVLG---RSFG--- : 407
 ATPT4 : TNSVSGEVKTQRRKRVAGPPVAYASAPFPFLPAPSFYSP : 431
 ATPT8 : --- : ---
 ATPT12 : ---LGLGIFVTA---LASGH--- : 387

Figure 1

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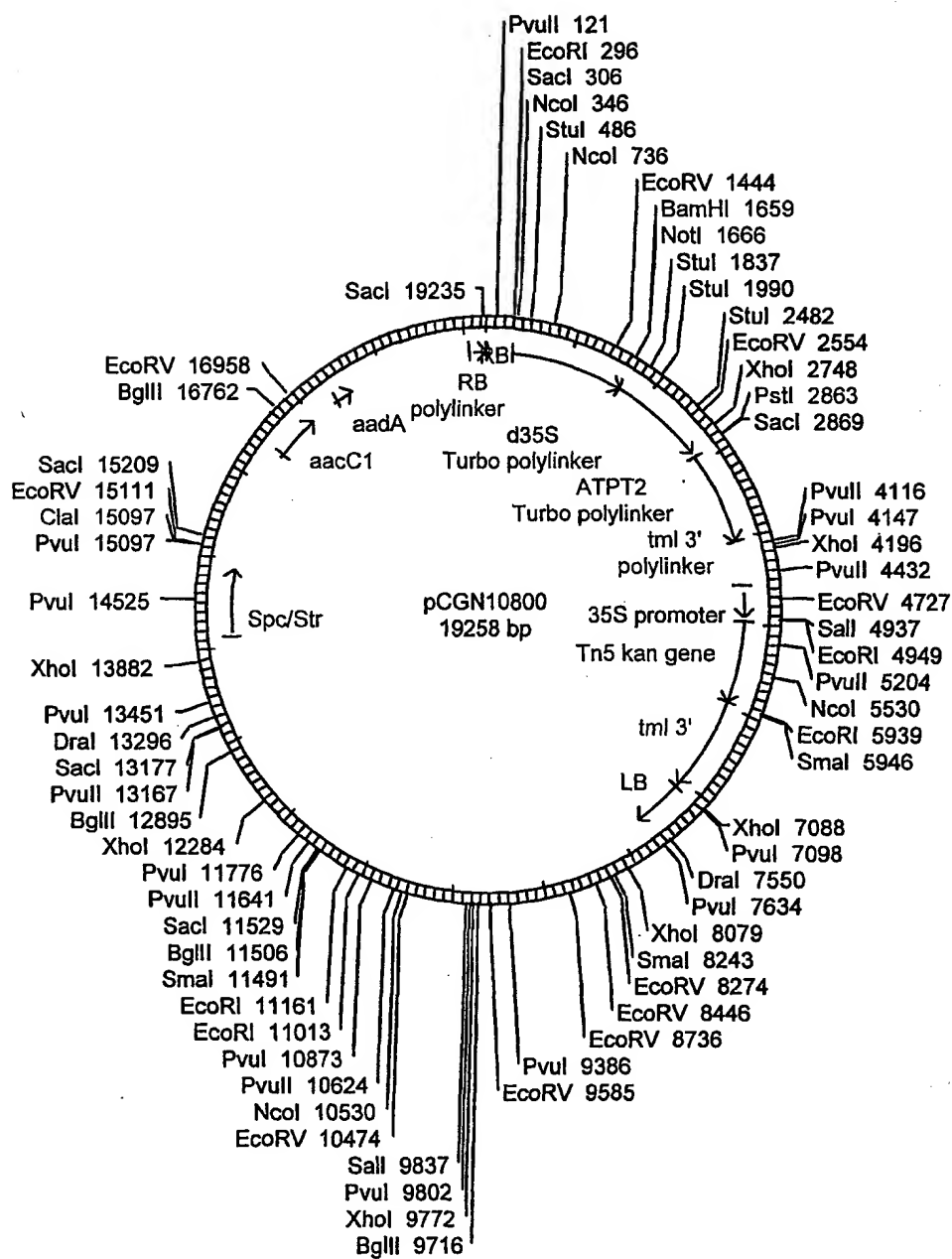


Figure 2

3/40

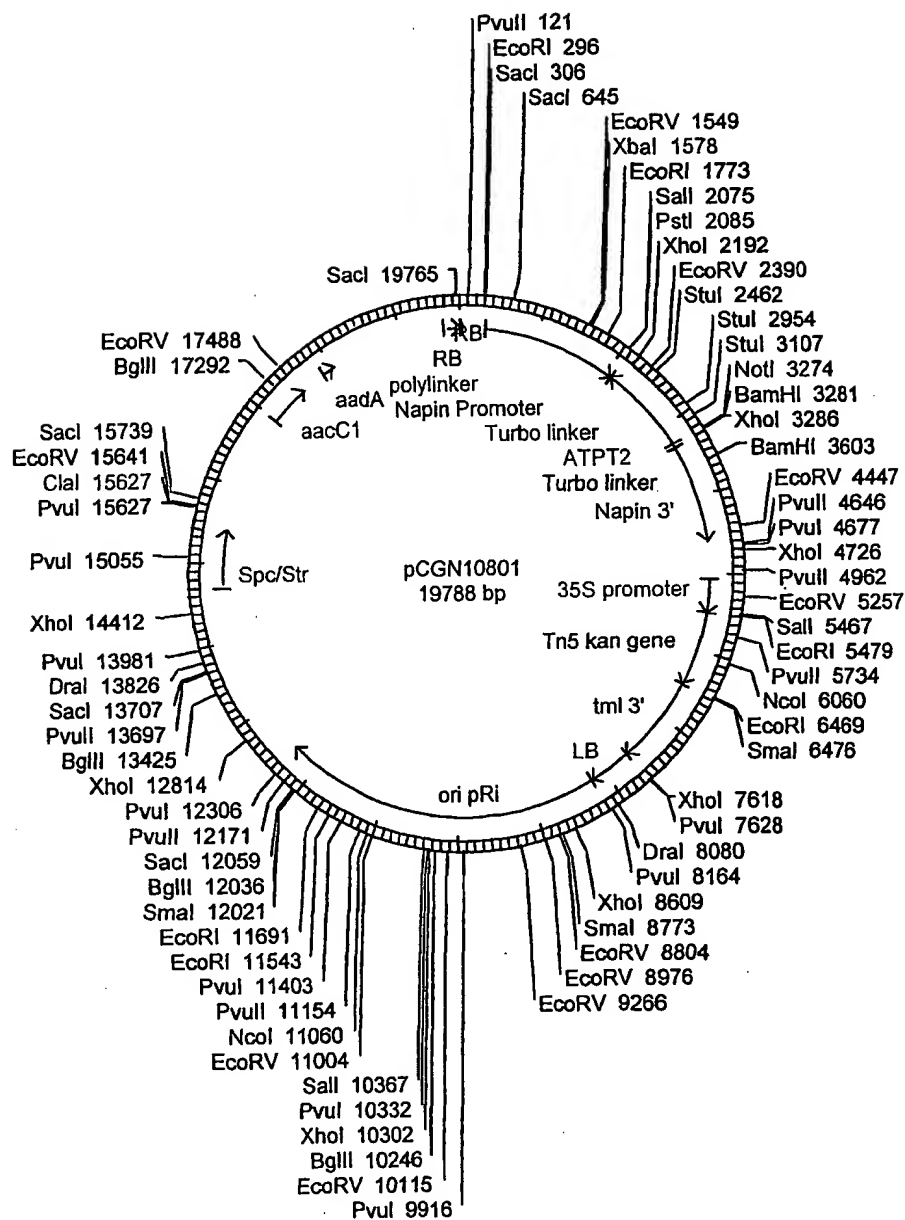


Figure 3

4/40

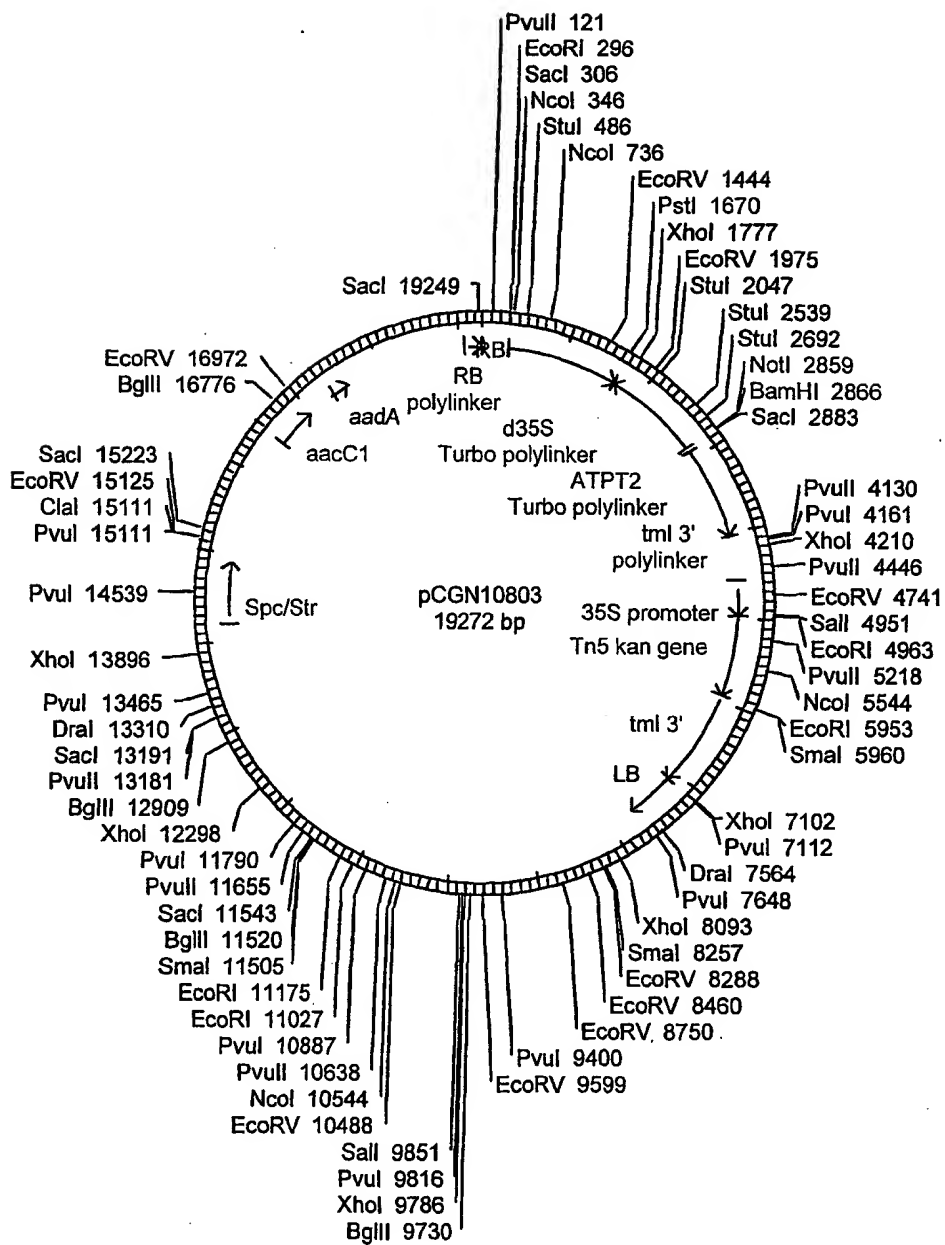


Figure 4

5/40

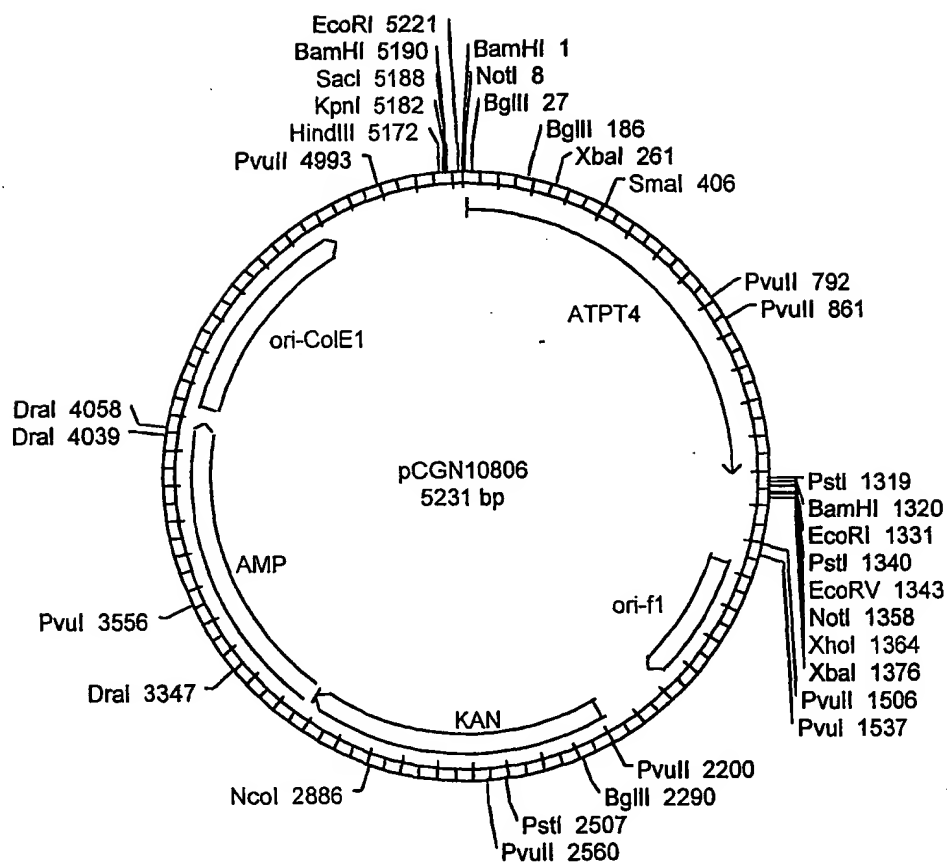


Figure 5

6/40

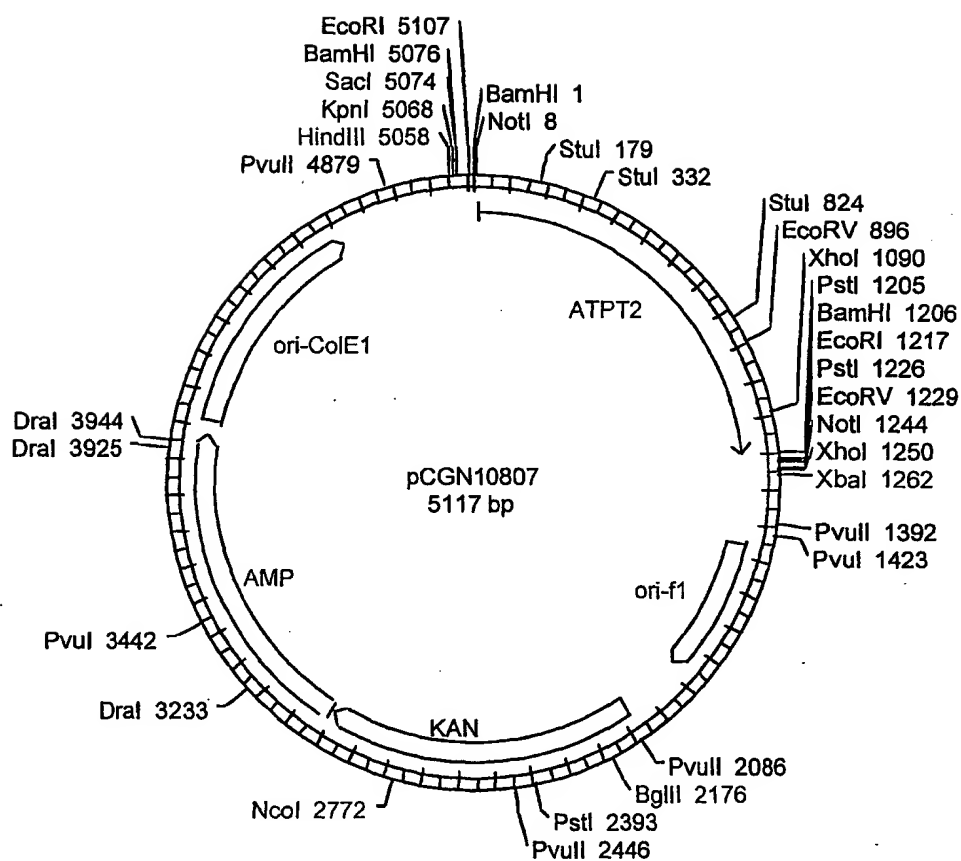


Figure 6

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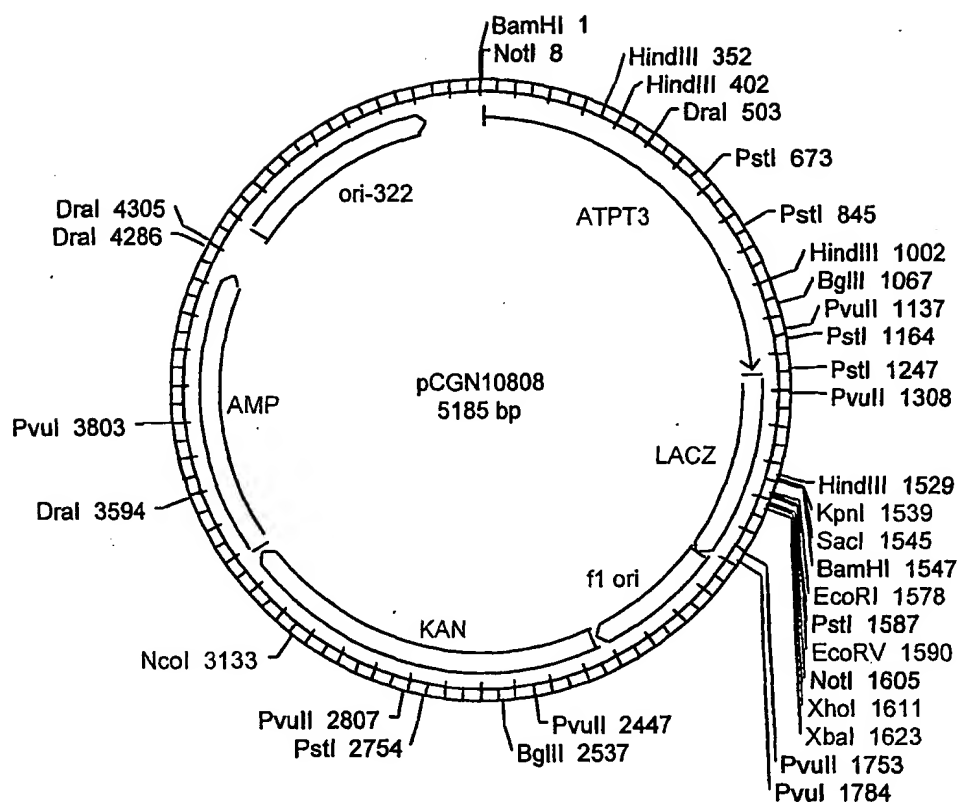


Figure 7

8/40

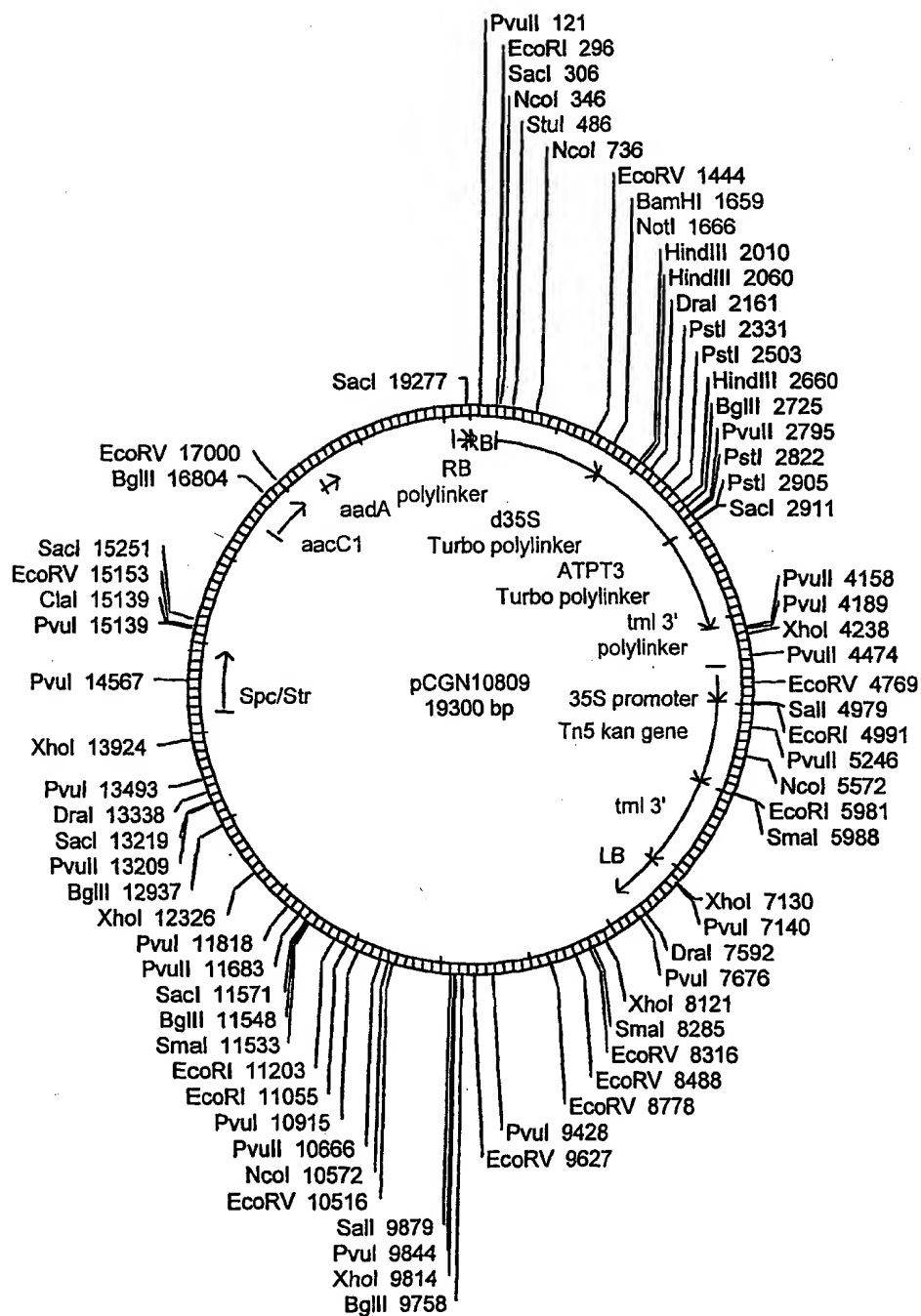


Figure 8

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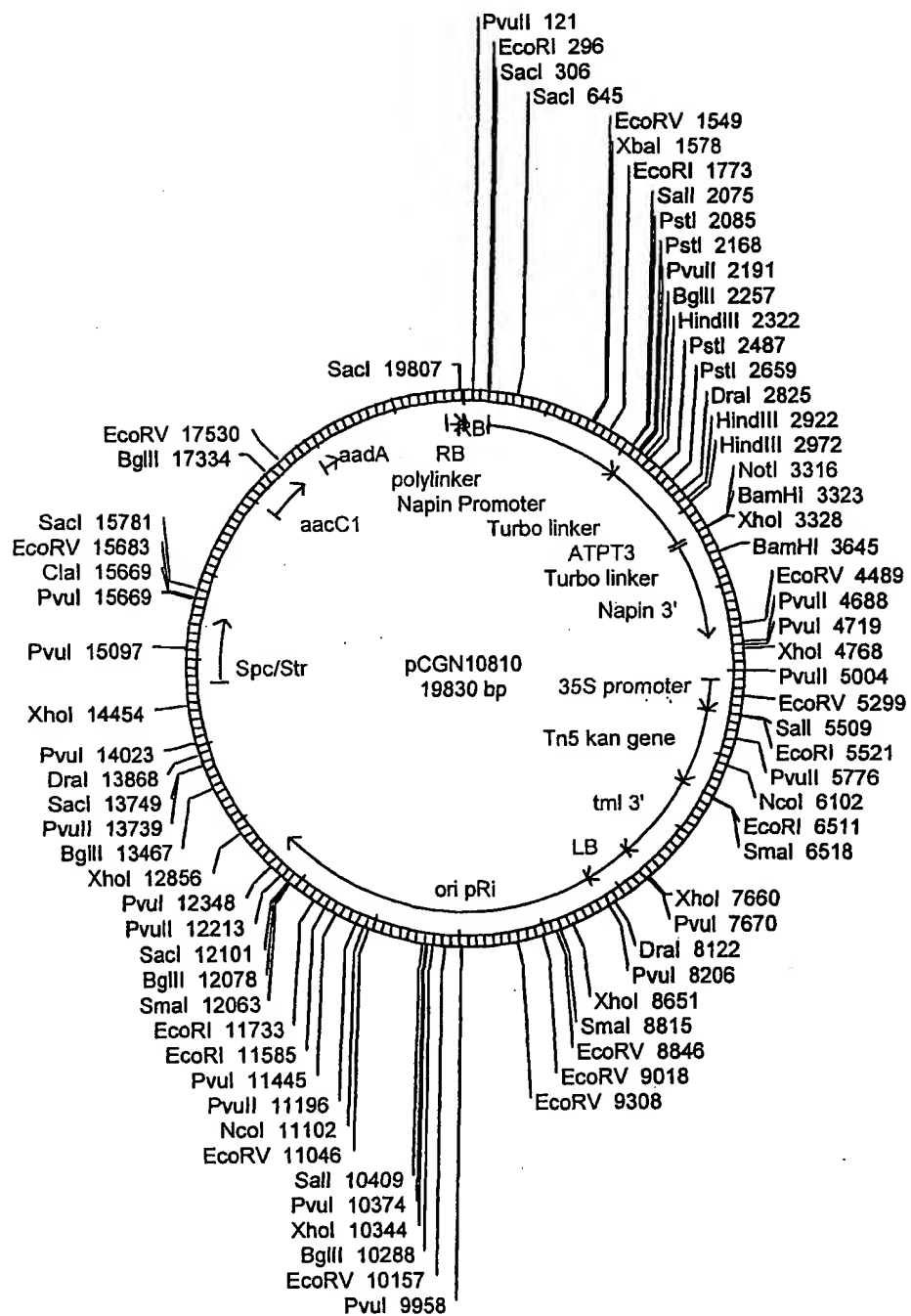


Figure 9

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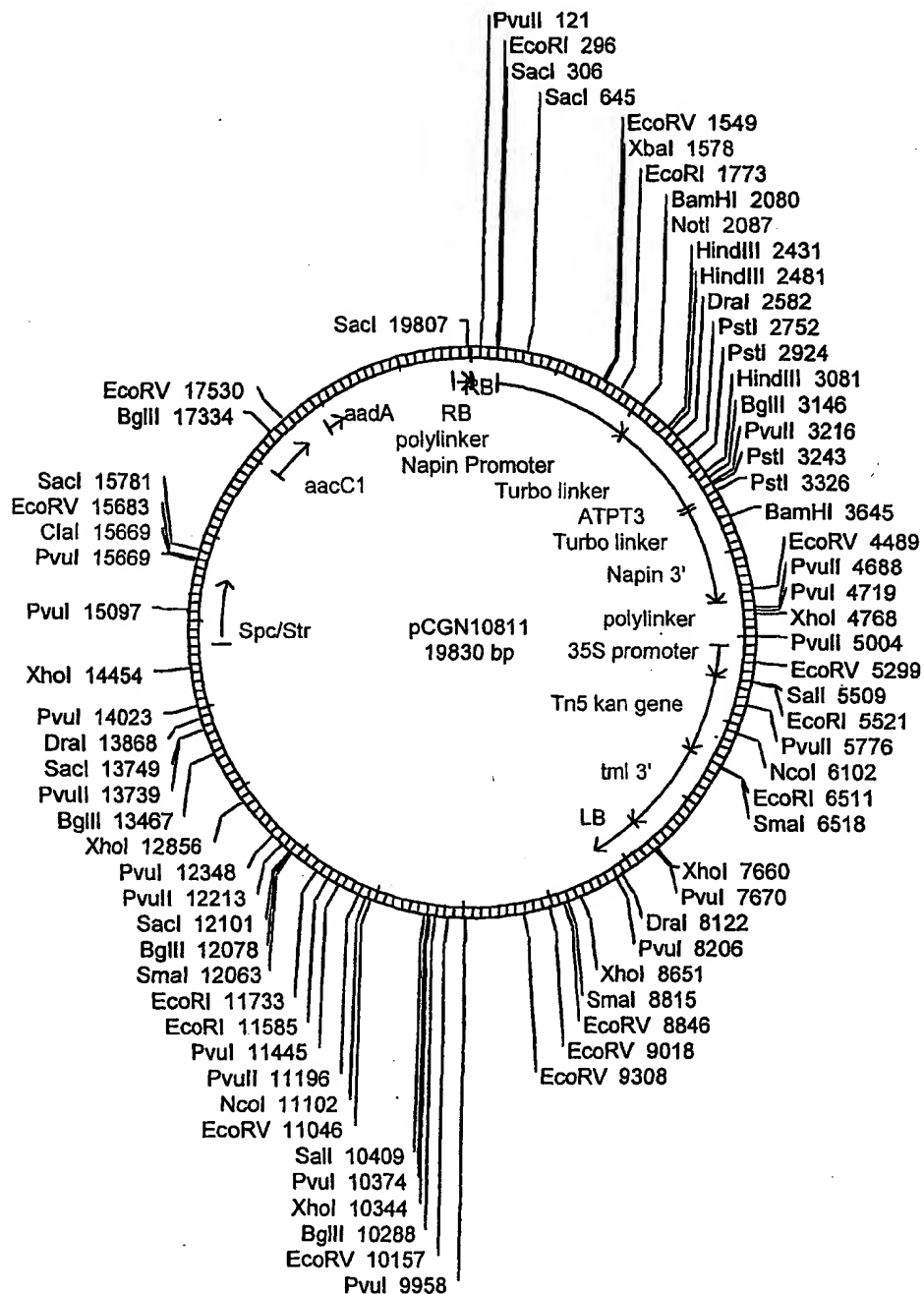


Figure 10

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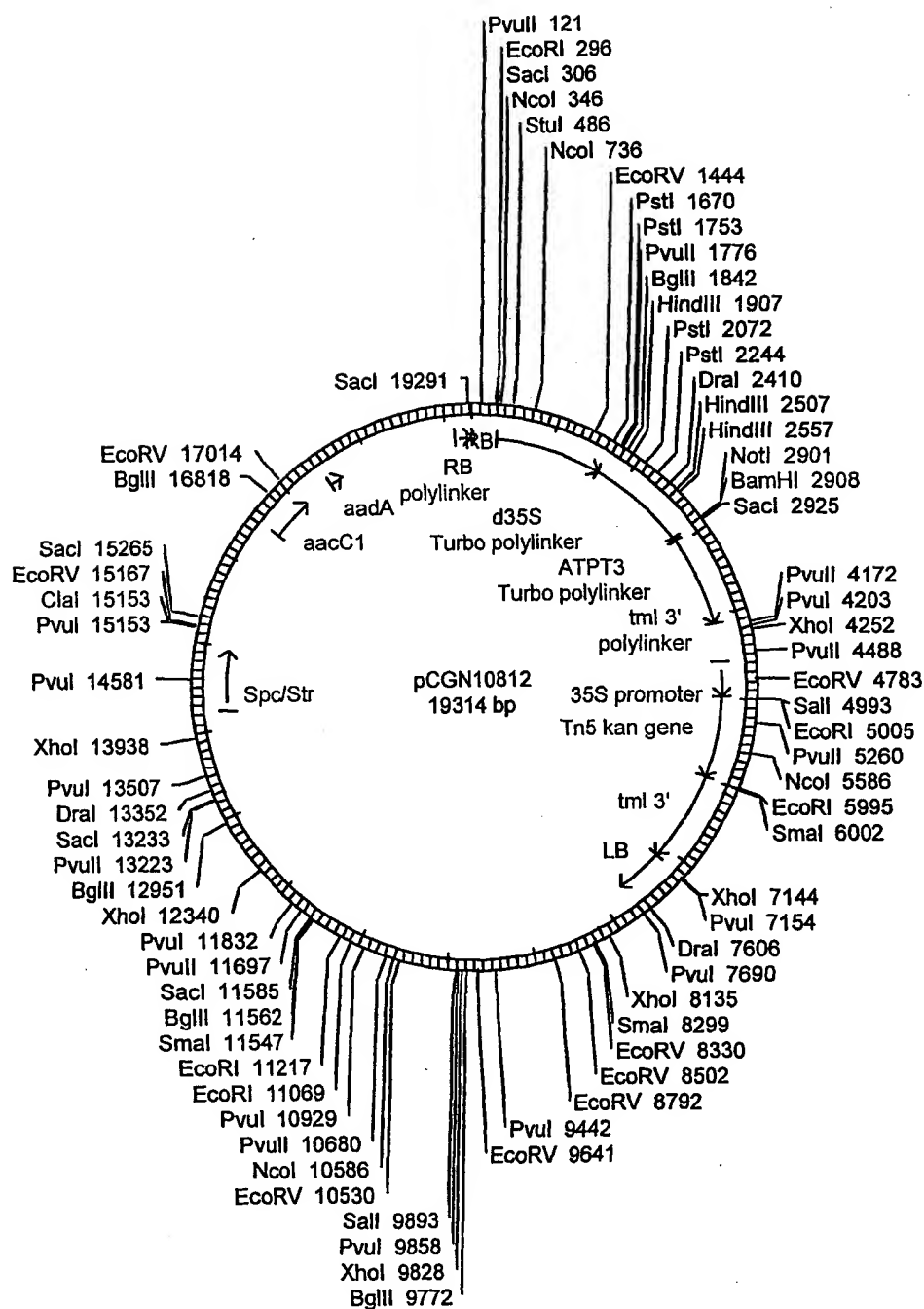


Figure 11

12/40

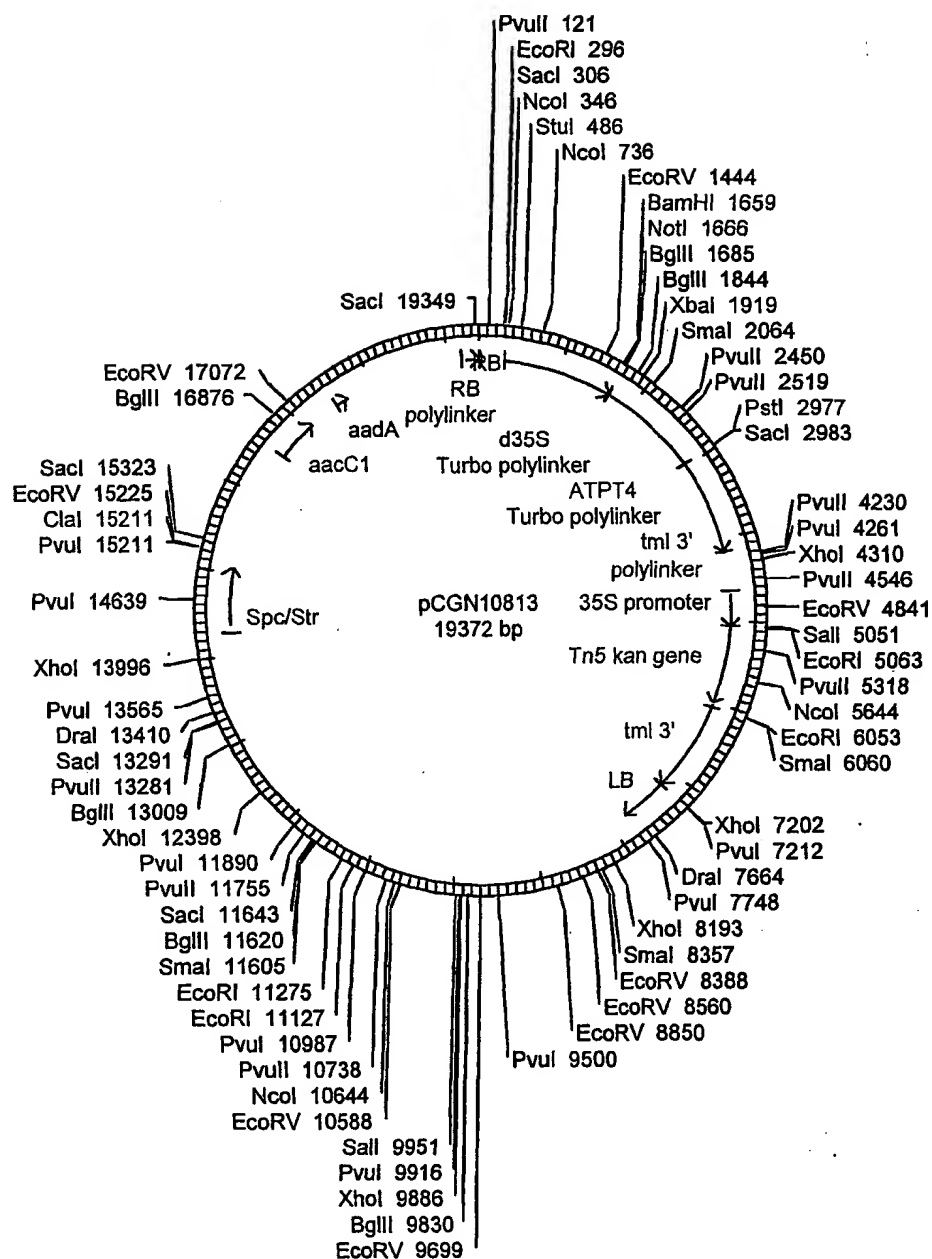


Figure 12

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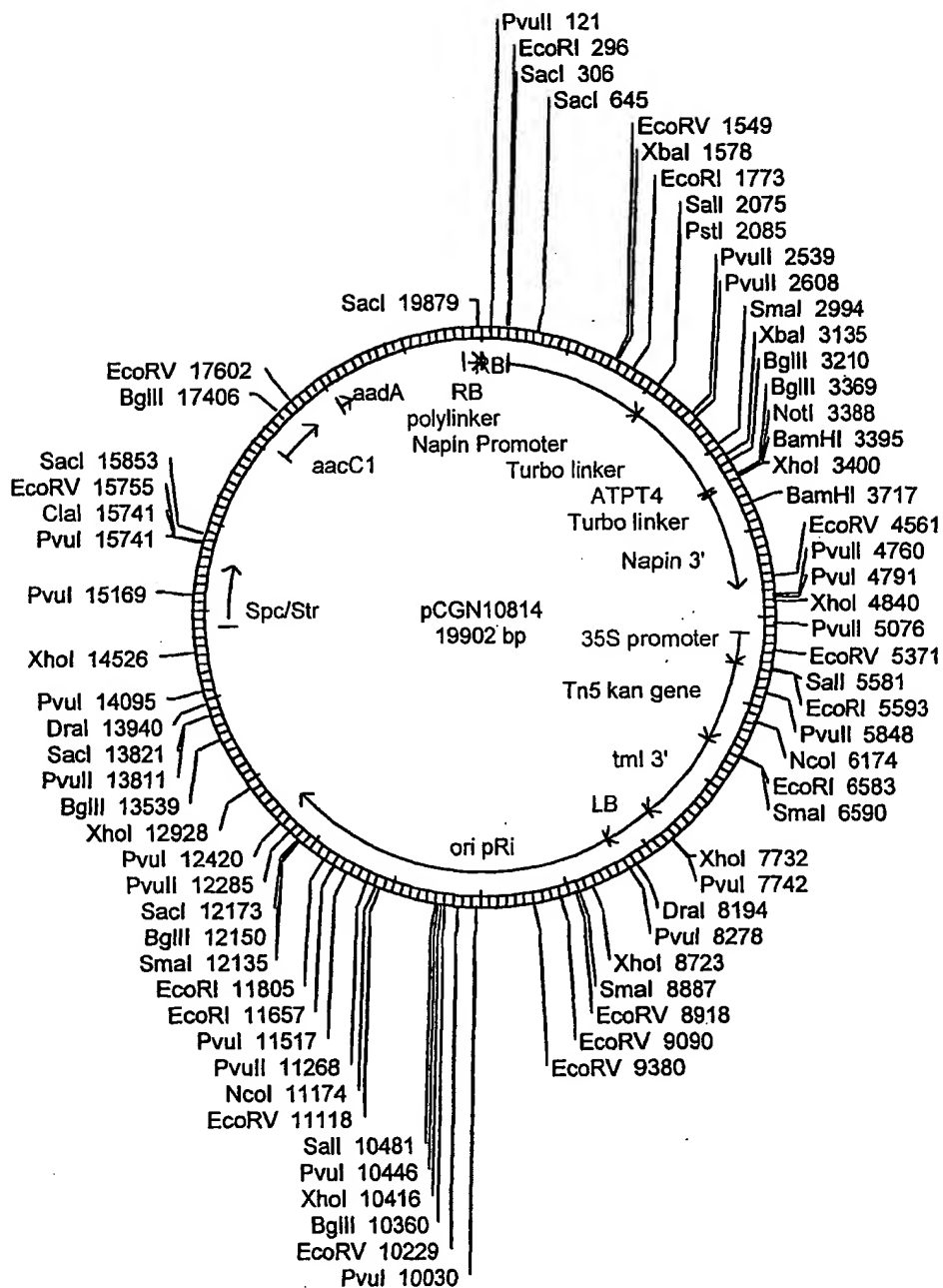


Figure 13

14/40

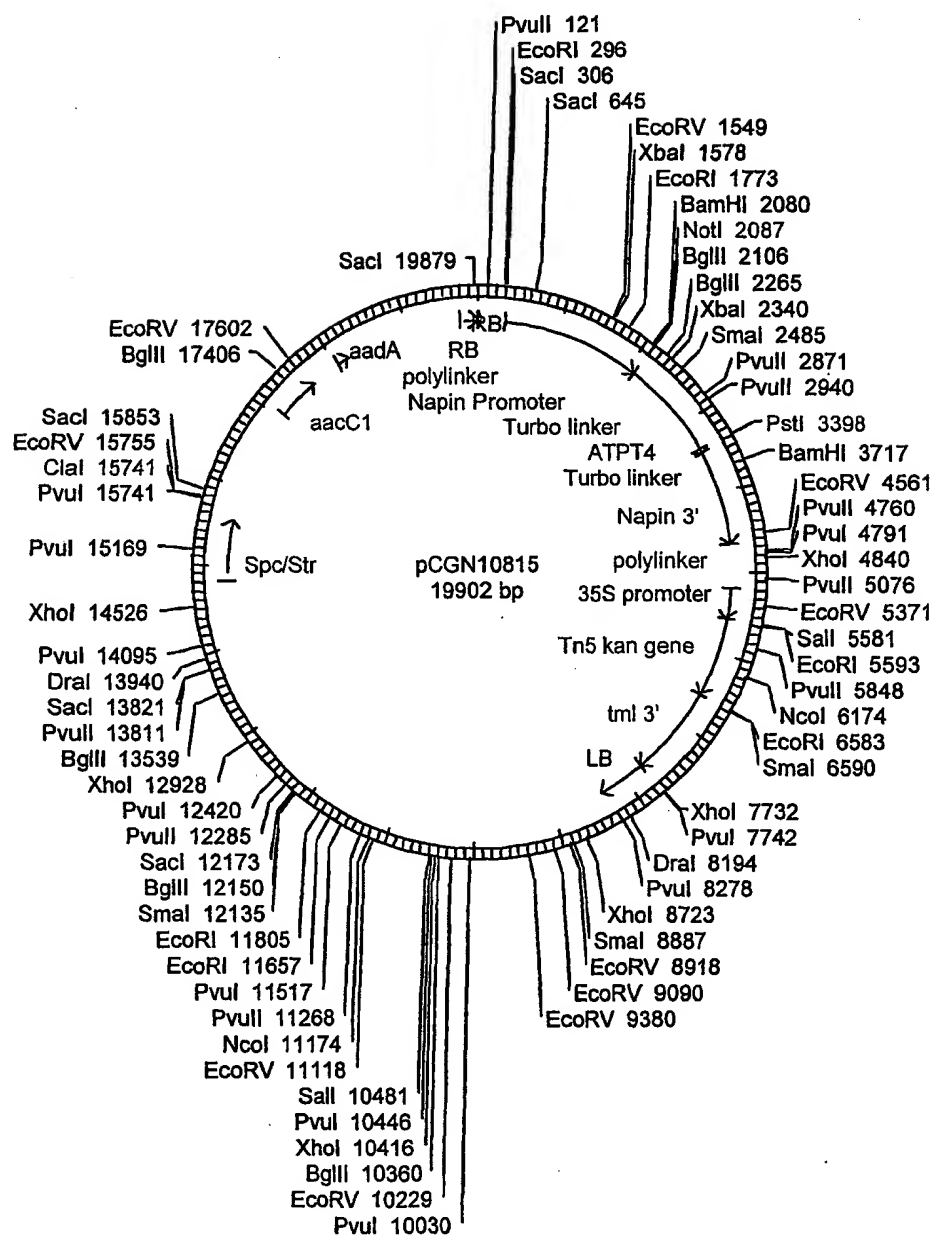


Figure 14

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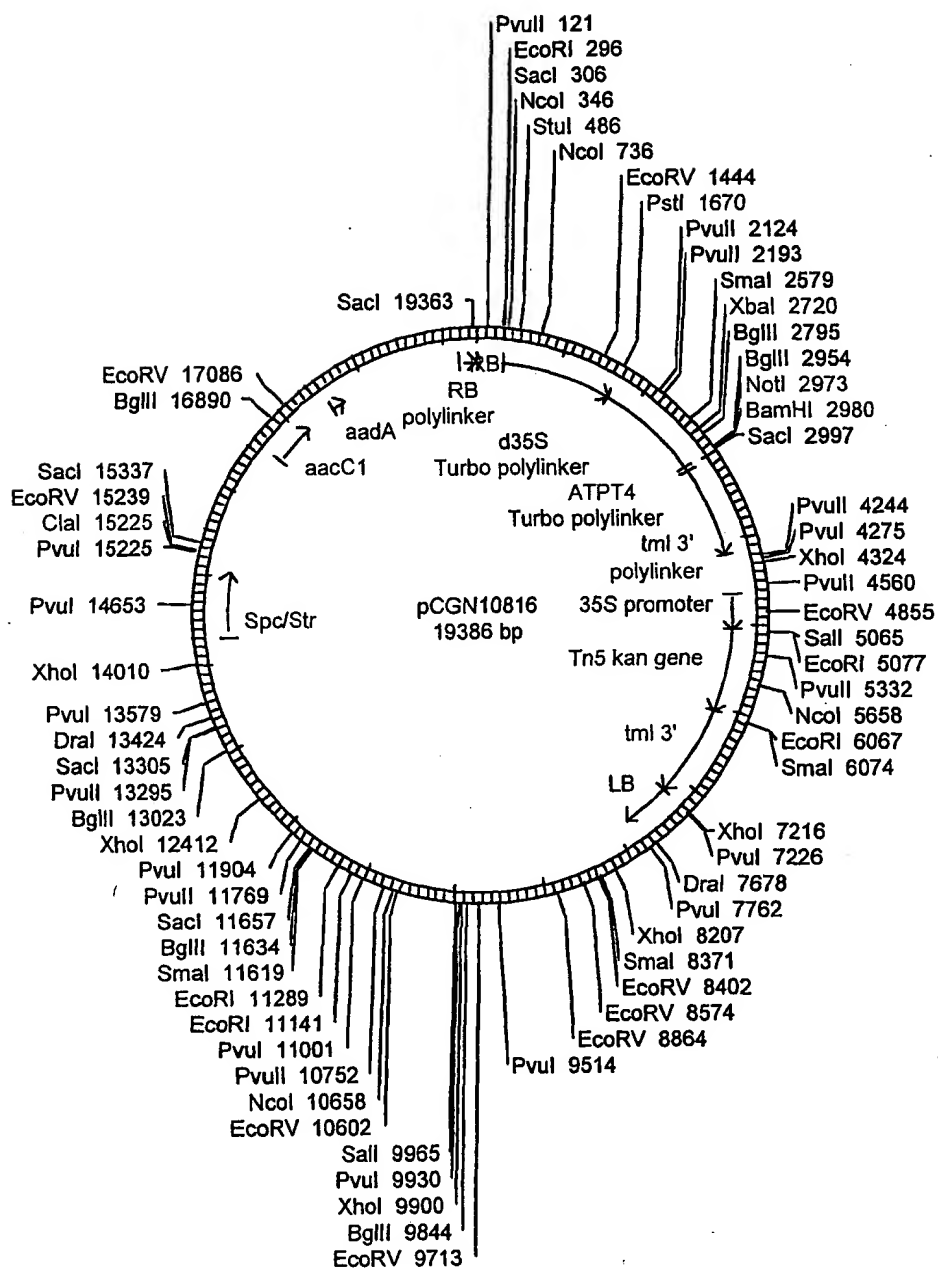


Figure 15

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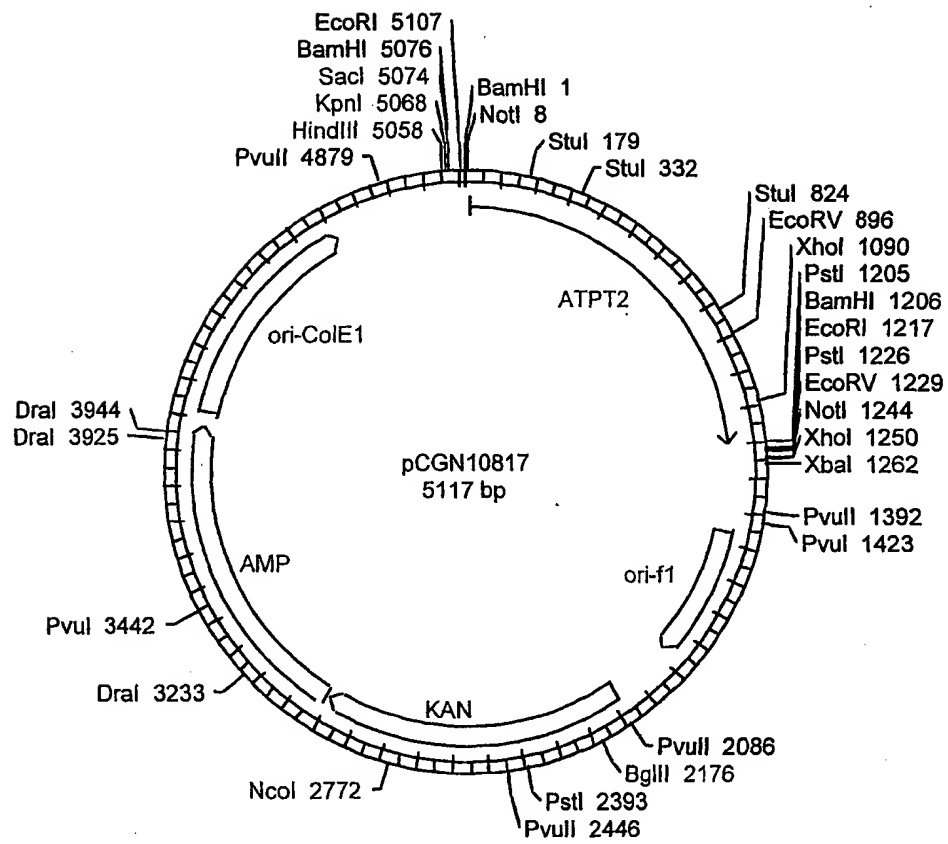


Figure 16

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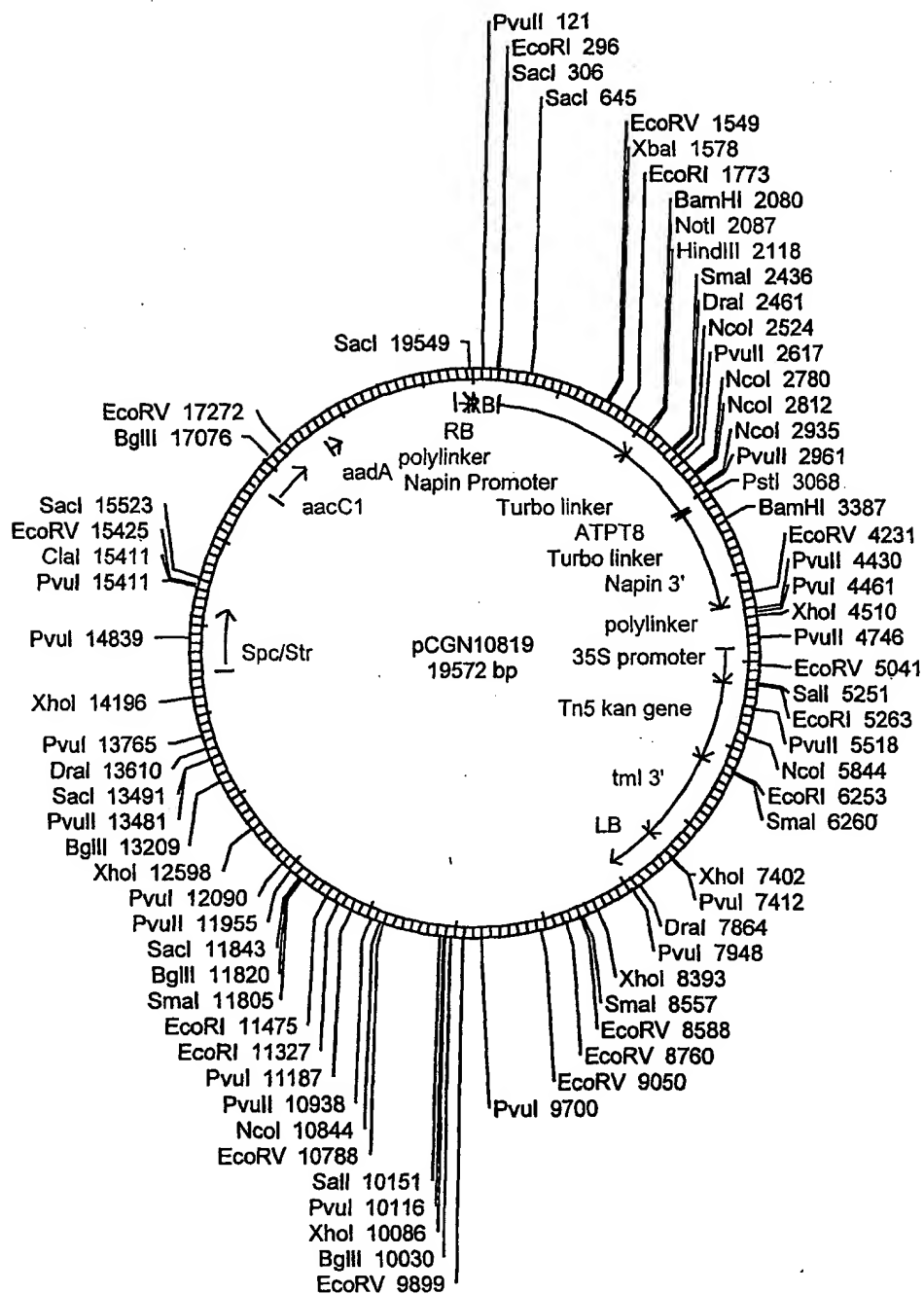


Figure 17

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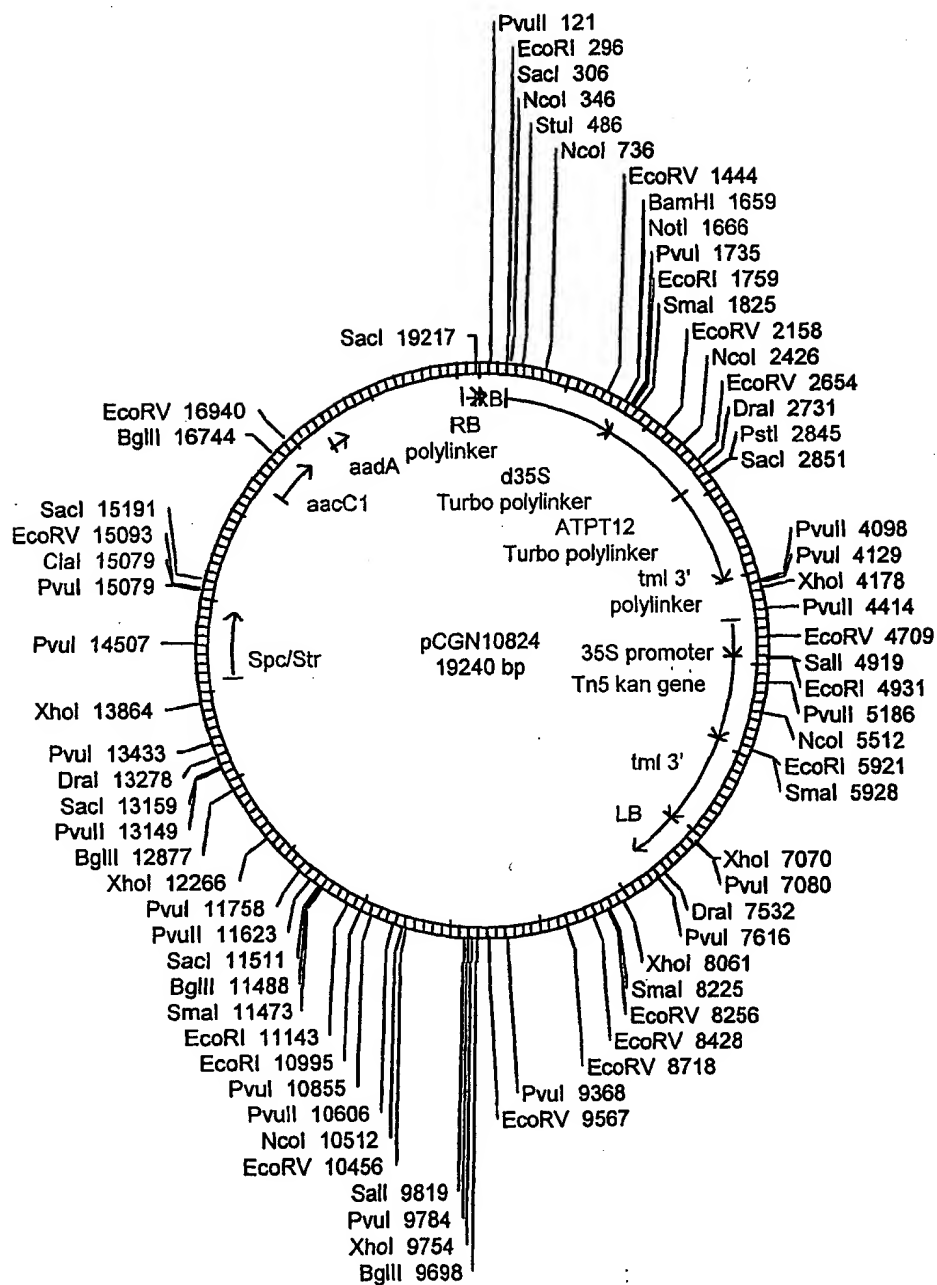


Figure 18

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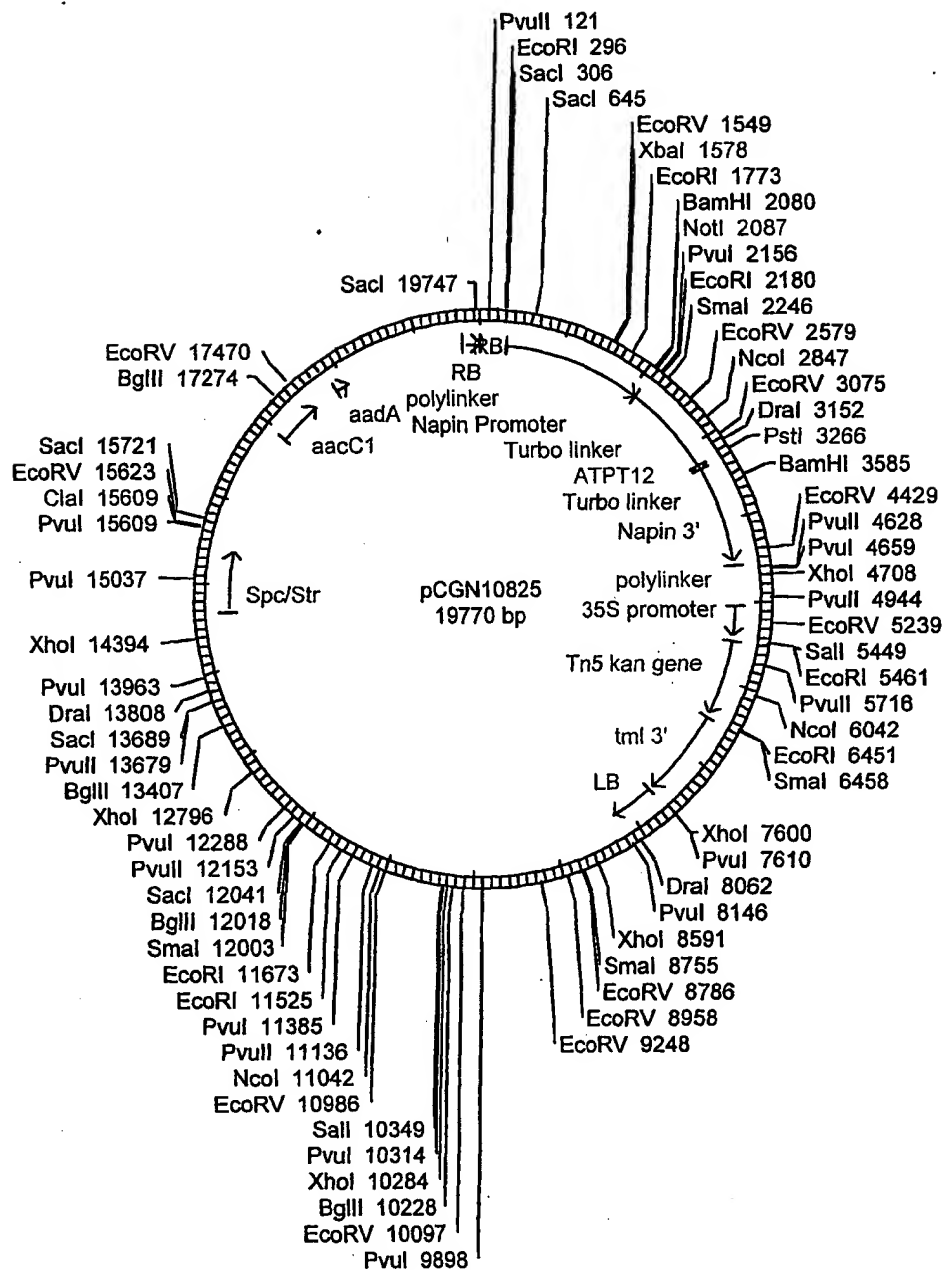


Figure 19

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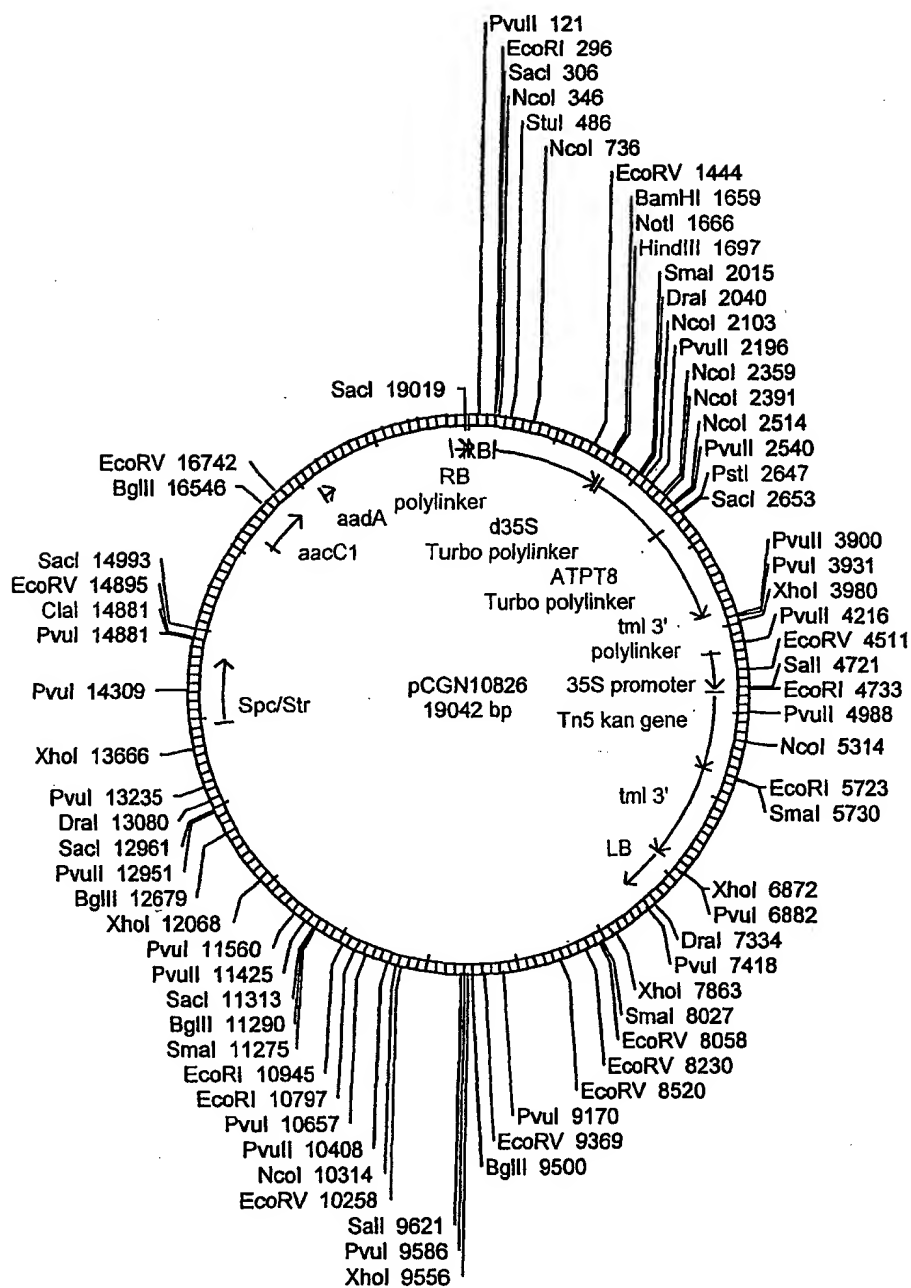


Figure 20

[illegible]

Figure 21

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	*	20	*	40	*	60	*	80	
ATPT2	:	-----	MESLLSSSLVSAAGGFCWKQKQNLKHLSELSEIRVLRCDSKVVAKPKFRNNLVRPDQGGSSLLLYPKHKSRFRVNATAGQ	:	80				
SIR1736	:	-----		:					
ATPT3	:	MAFFGLSVRRLLKSSVSVTPSSSSALLQSOHKSLSNPVTTHYTNFTKCYPSWNDNYQVMSKREHLOKKEFGVGMNYRLICGSSSS	:	89					
SIR0926	:	-----		:					
ATPT4	:	-----	WMRRSVVYRFSSRISVSSSLPNRPLIPWSRELCAVNSFSQP	:	67				
SIR1899	:	-----		:					
ATPT12	:	-----	MTSILNTVSTHSSRVSVDVRGVLSLRNSDSVEFT	:	63				
SIR0056	:	-----		:					
ATPT8	:	-----		:					
SIR1518	:	-----		:					

	*	100	*	120	*	140	*	160	*	1
ATPT2	:	PEAFDSNSKQK	:	-----	SFRDS	DAFYR	:	-----	FSRPH	VGTVLSLS
SIR1736	:	-----		:	-----	MATQAFWR	:	-----	FSRPH	IGTTLSSNAVY
ATPT3	:	SSVLEGPKKDDKEKSDGVVVKKASWIDLYLPEEVGRYAKLARLDKIGTWELAWPCNWS	:	-----	-----	-----	:	-----	-----	-----
SIR0926	:	-----		:	-----	PLMTIITYL	:	-----	LRWHK	PAGREILMIPADNA
ATPT4	:	TAAATATATTG	:	-----	EISSRVAALAG	GHHYAR	:	-----	CYWELSKAK	SMLVVATSG
SIR1899	:	-----		:	-----	AVCKSYQLTKP	:	-----	RIIPLL	HTTAASMI
ATPT12	:	DKVKSQTPDKAP	:	-----	AGGSSINQLLGH	KGAS	:	-----	QETNKKIR	LOLTKEVTWPPPLVGVVCG
SIR0056	:	-----		:	-----	ROLLGKGAAP	:	-----	GESSIKIR	QIMKEPIT
ATPT8	:	-----		:	-----	FFKRGVQKQF	:	-----	RSTILL	MATALNRVP
SIR1518	:	-----		:	-----	-----	:	-----	PMYTH	AVVPITVG

	*	200	*	220	*	240	*	260	
ATPT2	:	AVVAALMNTIYIVG	:	-----	-----	-----	:	-----	-----
SIR1736	:	AMVACLLGNVYIVG	:	-----	-----	-----	:	-----	-----
ATPT3	:	-----		:	-----	-----	:	-----	-----
SIR0926	:	-----		:	-----	-----	:	-----	-----
ATPT4	:	-----		:	-----	-----	:	-----	-----
SIR1899	:	-----		:	-----	-----	:	-----	-----
ATPT12	:	-----		:	-----	-----	:	-----	-----
SIR0056	:	-----		:	-----	-----	:	-----	-----
ATPT8	:	-----		:	-----	-----	:	-----	-----
SIR1518	:	-----		:	-----	-----	:	-----	-----

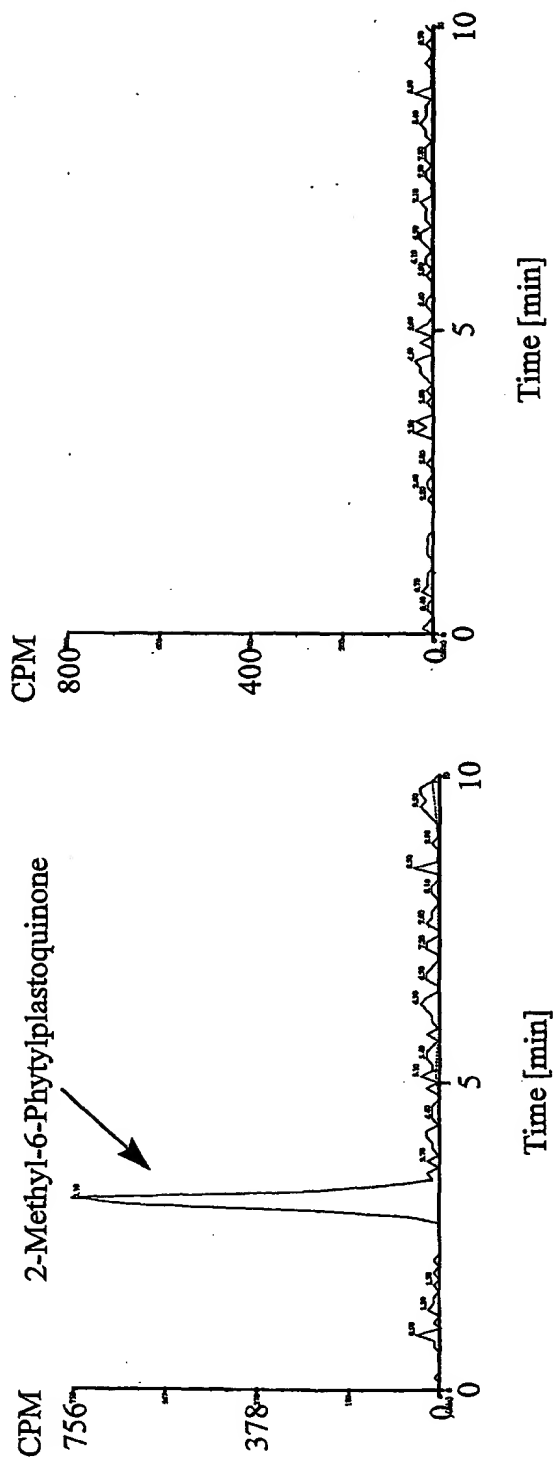
Figure 22A

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ATPT2	280	*	WKR-FALVAACILAVRAIIQIAFVH-IQTHVFGRPITFRP	300	*	320	*	340	*	313
SLR1736			LKR-FSLALALCILTIRGIVNGLFIP-FRIGLGYPTITITP							218
ATPT3			SIP-LMKRFTFWPOAFLGLTINWGAIG-IT--GSLAPS							328
SLR0926			AMP-GAKRVFPQLLSIAWGFVAHIS--NS--GATGDI							213
ATPT4			VMT-PLKQLHPINTWGAUVGAIPPIIG-MA--RASQHS							294
SLR1899			VNTHLKRHTAQNIVIGGAAGSIPPIIG-MA--VMTGDI							220
ATPT12			IS-APPLKLKQNGWGNFAGASYISLPWAGQITITP							308
SLR0056			IS-APPLKLKQNGWGNFAGASYIALPWAAGHAFETI							242
ATPT8			EITSSTEQRYSDMYMOKTYVKTASHGNSCKAVAILTQ							231
SLR1518			TQGPFRGLGYLGLGELICITITFGPLAI-AAAYISQ							223
										9
ATPT2	360	*	VFWTCVTLIQMAYAAIIVGATSPFIMSKVISVGHVIT	400	*	420	*	440	*	393
SLR1736			VFRGTHIITGTCYLAMAWGFWAAMPLNTAFVHSHLCL							304
ATPT3			KLMTGFGTASIGFALSQFSADLGWQYVASAAASGQ							407
SLR0926			GEAMGFFALTIGCIIFYTGMILMLNPLYLSIAHAB--							292
ATPT4			GKRIPAVARNCFYNIPIGFAIDWGLTSSWFCHESTT							379
SLR1899			VSOIHWYSILVVPFSLIIVYFHLQGLIYLAAITIT--							303
ATPT12			AKWICGANDITQLSVAGYILASGKPYVALAAVAL--							387
SLR0056			AAWHQVIMHDVFQAGIAGYILVHQQLYATITAILA--							324
ATPT8			ITAPILFAMEEFPQREAVDQVEKOPRNVDIAHEYJK							320
SLR1518			GSQVLTLSVNSLYLHTAGVICHQAPWQTLLEIASP							307
										9
ATPT2	460	*	-----	480	*	-----				
SLR1736			NTIF-----			308				
ATPT3			-----			-----				
SLR0926			-----			-----				
ATPT4			NQQLVEEAGLTNSVGEVKTQRRKRVQPPVAYASA			PPFLPAPSFYSP				431
SLR1899			-----			316				
ATPT12			-----			-----				
SLR0056			-----			-----				
ATPT8			K-----			321				
SLR1518			-----			-----				

Figure 22B

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Synechocystis 6803 wild type *Synechocystis* slr1736 knockout

Figure 23

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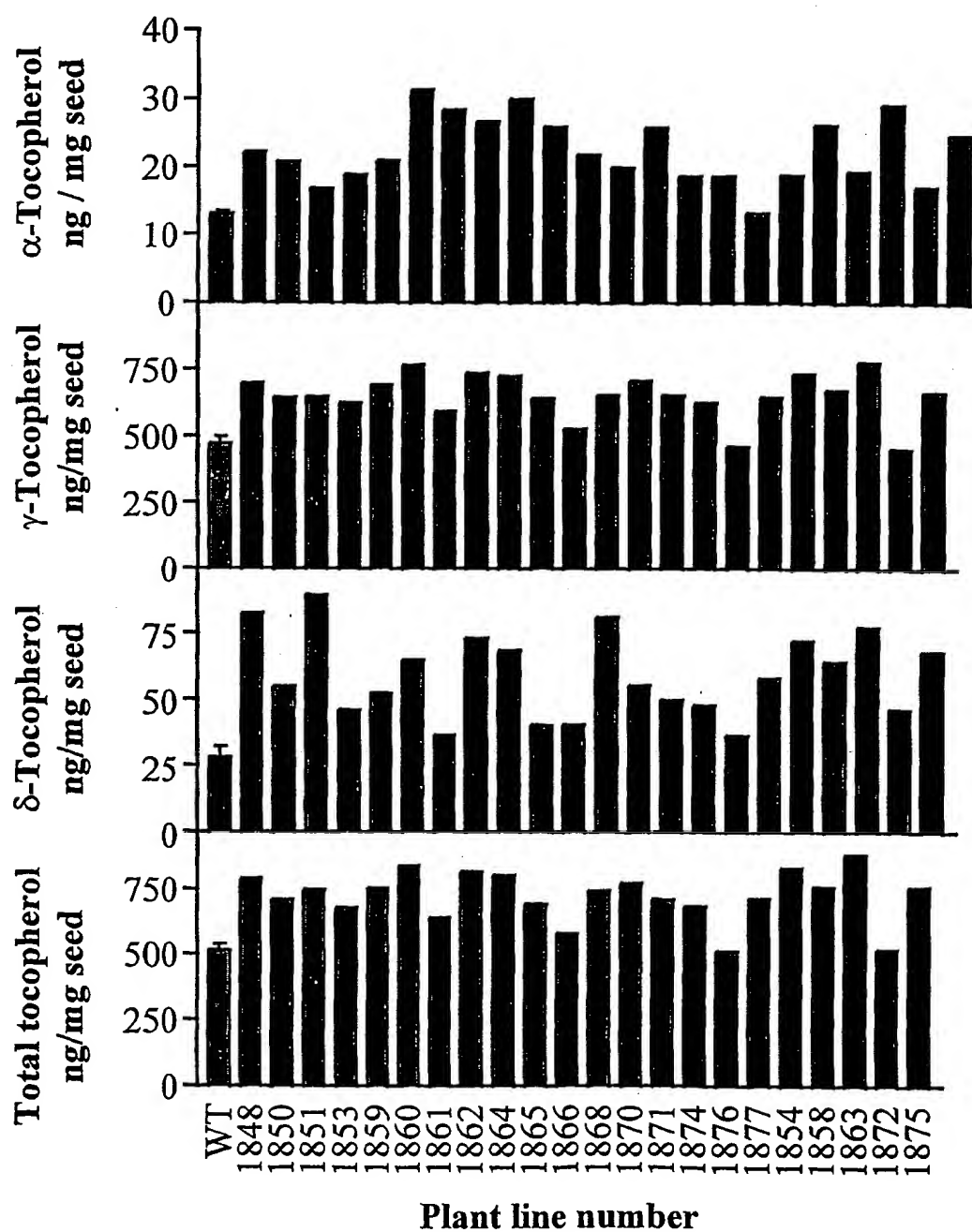


Figure 24

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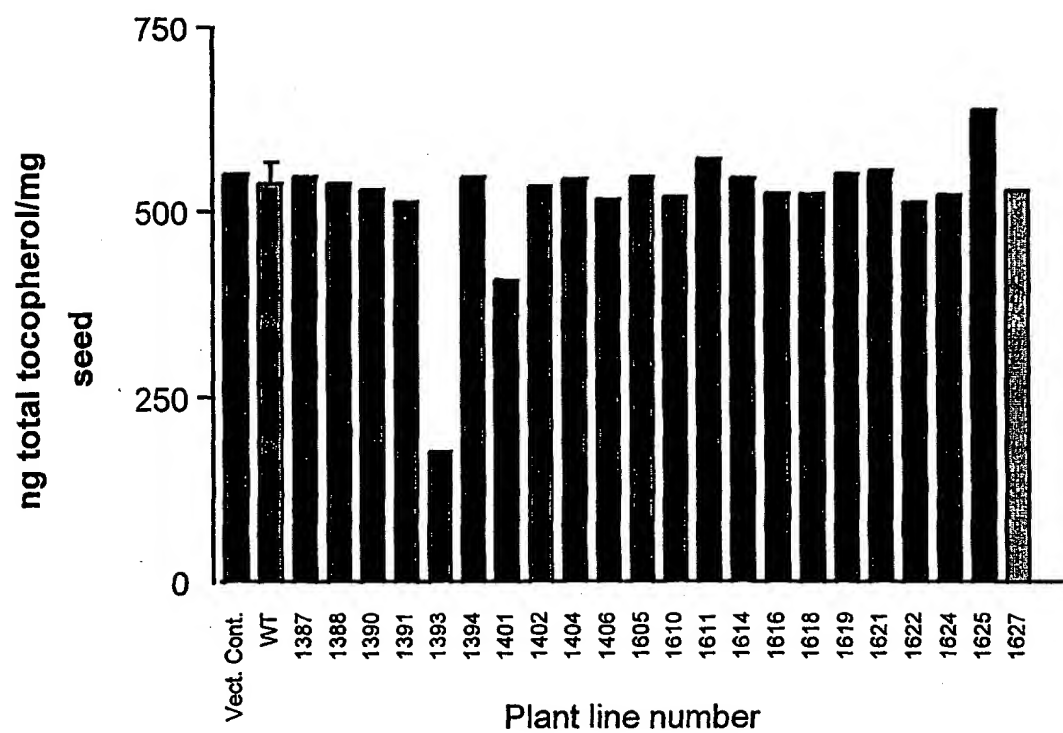


Figure 25

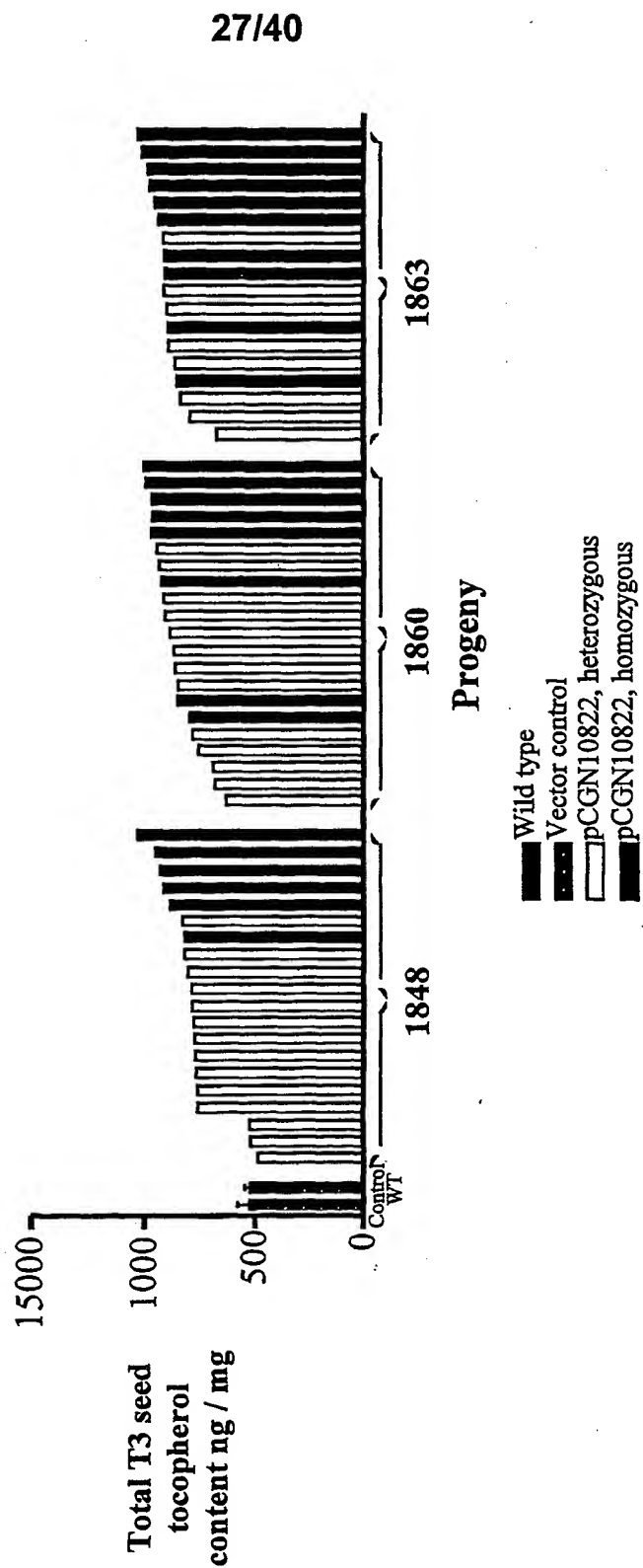


Figure 26

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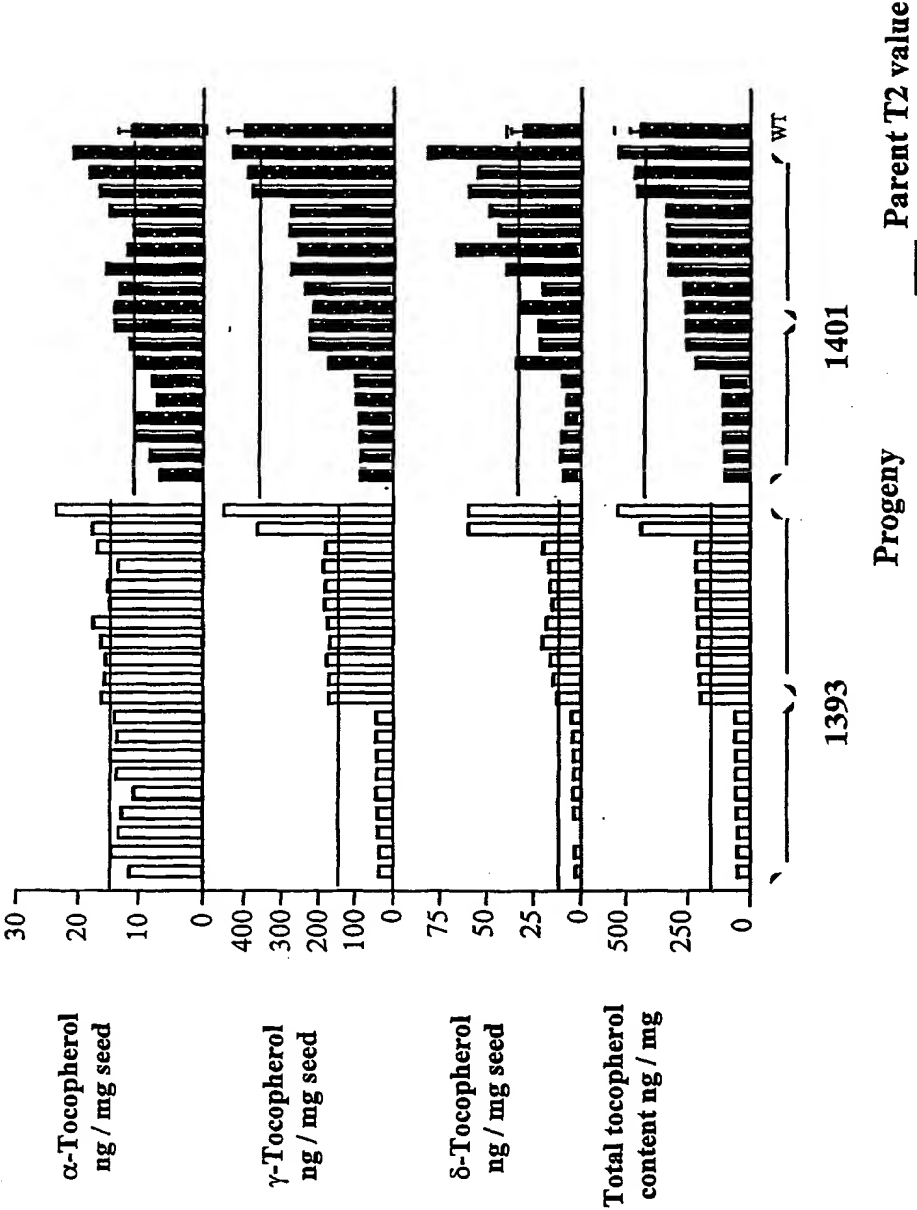
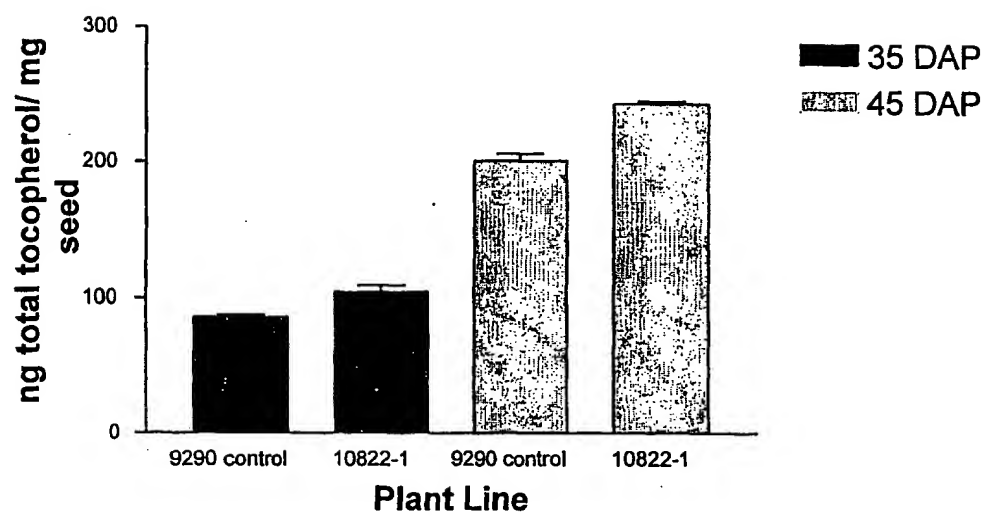


Figure 27

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Total tocopherol in Napin ATPT2 Canola Seed

**Figure 28**

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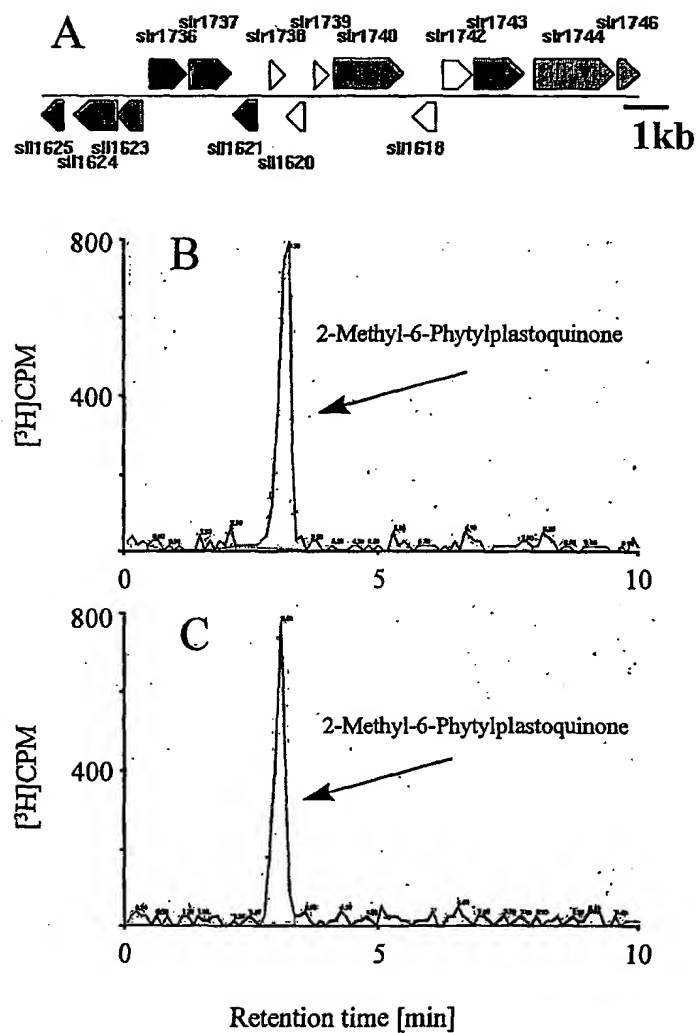


Figure 29

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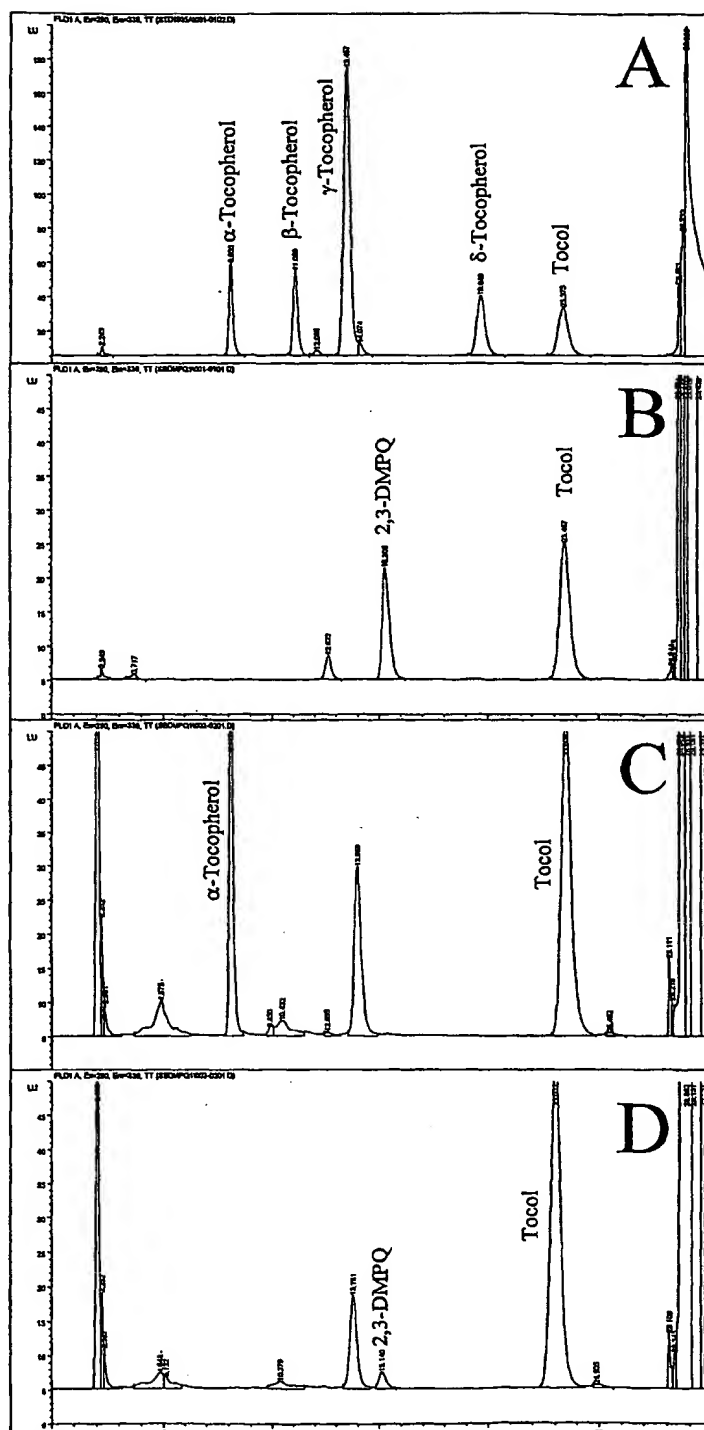


Figure 30

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Query Sequence: F4D11 AL022537
Database: PIR_T04448.atcea.list.fasta
Database: PIR_T04448
Plus (+) denotes forward strand, and minus (-) reverse strand.
Asterisks (*) denote bases not shown on pair wise alignments.

Alignment 1

```
Query-      12194 CACACGTTCTCGTCCTTTTCTTCTCTCTCTGCATTCTTCACAGAGTTTGTCACCACCA
genomic
ATCEA4C371+  1 ----- C est
Met
Query-      12134 ATGGA
ATCEA4C371+  2 ACCCCAAACATCACAAATTCACATTCTTTGCATATTTCTTCTTCTTCTCCATTATGGA

Query-      12075 GATACGGAGCTTGATTGTTTCTATGAACCCTAATTATCTTCCTTTGAGCTCTCTCGCCC
ATCEA4C371+  62 GATACGGAGCTTGATTGTTTCTATGAACCCTAATTATCTTCCTTTGAGCTCTCTCGCCC

Query-      12015 TGTATCTCTCTCACTCGCTCACTAGTTCCGTTCCGATCGACTAACTAGTTCCCGGCTC
ATCEA4C371+  122 TGTATCTCTCTCACTCGCTCACTAGTTCCGTTCCGATCGACTAACTAGTTCCCGGCTC

Query-      11955 CATTCTAGGGTTTCGATCTCCACCCCGAATAGTGAACTGACAAGATCTCCGT
ATCEA4C371+  182 CATTCTAGGGTTTCGGCGTCGATCTCCACCCCGAATAGTGAACTGACAAGATCTCCGT

Query-      11895 TAAACCTGTTTACGTCCCGACGCTCTCCCAATCGCGAATCCGGACTCTTCACAGTGGTA
ATCEA4C371+  242 TAAACCTGTTTACGTCCCGACGCTCTCCCAATCGCGAATCCGGACTCTTCACAGTGG
Synecho seq aligns from
here

Query-      11835 AATTGATCCATTCCATTCTCTTCTTGTGTTTATTAAAGCTCCAATTTCAG
ATCEA4C371+  299
~~ 60 bp removed ~~

Query-      11715 *****TTTG
ATCEA4C371+  299
PIR:T04448  1 -----

Query-      11655 GTGGCTCACCATTGACGACTACTTTTGAATTGAGTTTGGAAAAATGCAATTAAACAT
ATCEA4C371+  299
PIR:T04448  1 -----
M Q F N I
arab sequence which is incorrect
```

Figure 31A

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```
Query- 11595 CAGAGAGTTTTTTTTTTTATGGTTGATAACTTATTGTTTAACTTTTGAAAAATGCAGATN
ATCEA4C371+ 299 -----ATA
PIR:T04448 6 R E F F L W L I T Y C L T F E K C R Y

Query- 11535 CCATTTCGATGGAACACCTCGGAAGTTCTTCGAGGGATGGTATTTTCAGGGTTTCCATCCC
ATCEA4C371+ 302 CCATTTCGATGGAACACCTCGGAAGTTCTTCGAGGGATGGTATTTTC TCCATCCC
PIR:T04448 26 H F D G T P R K F F E G W Y F S I P

Query- 11475 AGAGAAGAGGGAGAGTTTTTGTATTATGTTATCTGTGGAGAATCCTGCATTTTCGGCAGAG
ATCEA4C371+ 362 AGAGAAGAGGGAGAGTTTTTGTATTATGTTATCTGTGGAGAATCCTGCATTTTCGGCAGAG
PIR:T04448 46 E K R E S F C F M Y S V E N P A F R Q S

Query- 11415 TTTGTCACCATTGGAAGTGGCTCTATATGGACCTAGATTCACTGGTGTGGAGCTCAGAT
ATCEA4C371+ 422 TTTGTCACCATTGGAAGTGGCTCTATATGGACCTAGATTCACTGGTGTGGAGCTCAGAT
PIR:T04448 66 L S P L E V A L Y G P R F T G V G A Q I

Query- 11355 TCTTGGCGCTAATGATAAATATTTATGCCAATACGAACAAGACTCTCACAATTTCTGGGG
ATCEA4C371+ 482 TCTTGGCGCTAATGATAAATATTTATGCCAATACGAACAAGACTCTCACAATTTCT
PIR:T04448 86 L G A N D K Y L C Q Y E Q D S H N F W G
ATCEA4C371+ Exon 11538 11301 Confidence: 100 100

Query- 11295 AGGTAACCTCTGACCCTTAAATGCTGTGTCATGACAATAAGAAATCATATCTGAGTCT
ATCEA4C371+ 537 -----
PIR:T04448 106 D
PIR:T04448 Exon 11609 11294 Confidence: 100 100

Query- 11235 TTTCTCTACTTCTAGTACTAATGTTTCGTTATTGTTGTTAAAGATCTAAGTCTTATCTGAA
PIR:T04448 107 -----

Query- 11175 TTTTGTACATTTTGGTTCTGGTGCTTTCTCAACATGAATTTGTATATATGACTTTAAAG
PIR:T04448 107 -----

Query- 11115 ATTGCTTACCTAAAGTTTTTACTCATGATAGATCGACATGAGCTAGTTTGGGGAATAC
PIR:T04448 107 -----R H E L V L G N T

Query- 11055 TTTTGTGCTGTGCCAGGCGCAAAGGCTCCAAACAAGGAGGTTCCACCAGAGGTTCTCAC
PIR:T04448 116 F S A V P G A K A P N K E V P P E
PIR:T04448 Exon 11083 11004 Confidence: 96 100
```

Figure 31B

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```
Query- 10995 TCCTCCCTTGTGGTTACTTTGTTATCTGTTAAATAGTTTCCAAATTGTATCCGGATAGT
PIR:T04448 133

Query- 10935 GTTCTACTTCTCCTTGTAGAAAATCTCAAGTTTTTGTACTCTTGCTATTCTCTGGATG
PIR:T04448 133

Query- 10875 TTGATTGTAAAGCATGTCGTTTATTGTAGGAATTTAACAGAAGAGTGTCCGAAGGGTT
PIR:T04448 133 E F N R R V S E G F

Query- 10815 CCAAGCTACTCCATTTTGGCATCAAGGTCACATTGCGATGATGGCCGGTAATTATATGA
PIR:T04448 143 Q A T P F W H Q G H I C D D G R
PIR:T04448 Exon 10844 10768 Confidence: 100 100

Query- 10755 TTCTATGCACAACAAGAATTCACATATATTATAAATATTGGATATTGAGTATTTTGTGTA
PIR:T04448 159

Query- 10695 AAATTTCTGTGTTTAAATCTGACTTGACTTGTGTCAGTACTGACTATGCGGAAACTG
PIR:T04448 159 T D Y A E T V

Query- 10635 TGAAATCTGCTCGTTGGGAGTATAGTACTCGTCCCGTTTACGGTTGGGGTGATGTTGGGG
PIR:T04448 166 K S A R W E Y S T R P V Y G W G D V G A

Query- 10575 CCAAACAGAAGTCAACTGCAGGCTGGCCTGCAGCTTTTCTGTATTGAGCCTCATTGGC
PIR:T04448 186 K Q K S T A G W P A A F P V F E P H W Q

Query- 10515 AGATATGCATGGCAGGAGGCTTTCCACAGGTGTGAGCTTTGCTTGATTGACTTAAAGTT
PIR:T04448 206 I C M A G G L S T G
PIR:T04448 Exon 10655 10486 Confidence: 96 100

Query- 10455 AATAAATAGACGGTTAAGTTTACTTGCTTAGTACTAACAGAAAATTAAGAAAGAAACCAC
PIR:T04448 216

Query- 10395 CCTCTTCTATCAGCAGAACTGCTATTGTAGTTCTTATTTTCTCTTGTATTGTCAGG
PIR:T04448 216

Query- 10335 GTGGATAGATGGGGCGGTGAAAGGTTTGAGTTTCGGGATGCACCTTCTTATTCAGAGAA
PIR:T04448 216 W I E W G G E R F E F R D A P S Y S E K

Query- 10275 GAATTGGGGTGGAGGCTTCCCAAGAAAATGGTTTTGGGTAACATTTCATCCCTTTTGCT
PIR:T04448 236 N W G G G F P R K W F W
PIR:T04448 Exon 10336 10239 Confidence: 96 100
```

Figure 31C

35/40

```
Query-      10215 ACATTCTTGTGCACTTTAGTTAGCTAGTGGACCTGTGTATACACCCACATGTAGTA
PIR:T04448      248 -----

Query-      10155 TACTTGTTTGATAGCTTTATTTGTCAATGTCTCTTTACAGGTCCAGTGTAAATGTCTTTGA
PIR:T04448      248 ----- V Q C N V F E

Query-      10095 AGGGGCAACTGGAGAAGTTGCTTTAACCAGGTGGCGGGTTGAGGCAATTGCCTGGATT
PIR:T04448      255 G A T G E V A L T A G G G L R Q L P G L

Query-      10035 GACTGAGACCTATGAAAATGCTGCACTGGTATGCACCTATAAGATCTTCTTAAGCAATGA
PIR:T04448      275 T E T Y E N A A L
PIR:T04448      Exon 10115 10008 Confidence: 100 100

Query-      9975 CAGTGAGTATTAGAAGGCAGATAGTTTACAAAAGCTCTGGGCCCTTGTAATCTGCAGGT
PIR:T04448      284 ----- V

Query-      9915 TTGTGTACACTATGATGGAAAAATGTACGAGTTTGTTCCTTGGAAATGGTGTGTAGATG
PIR:T04448      285 C V H Y D G K M Y E F V P W N G V V R W
GSDB:S:495-    532 ----- tagatg

Query-      9855 GGAAATGTCTCCCTGGGG TTATTTGGTATATAACTGCAGAGAACGAAAACCATGTGGTAA
PIR:T04448      305 E M S P W G Y W Y I T A E N E N H V
GSDB:S:495-    526 ggaat tctccctgggggttattggtatataactgcagagaNcgNaaaccatgtg
PIR:T04448      Exon 9917 9801 Confidence: 100 100
GSDB:S:495-    Exon 9861 9801 Confidence: 93 93

Query-      9796 ATTTGTTTTACTAGTTTCATTGAGTTTACTTTTGACATCATATCATTCCCTTATGGCTA
PIR:T04448      323 -----
GSDB:S:495-    471 -----

Query-      9736 GATTCCAACACCCGATGAATGCTTTGTGACAGGTGGAAGTAGAGGCAAGAACAATGAAG
PIR:T04448      323 ----- V E L E A R T N E A
GSDB:S:495-    471 ----- gtggaactagaggcNagaacaaatgaag

Query-      9676 CGGGTACACCTCTGCGTGCTCTACACAGAAGTTGGGCTAGCTACGGCTTGACAGATA
PIR:T04448      333 G T P L R A P T T E V G L A T A C R D S
GSDB:S:495-    443 cgggtacacctctgctgctctctaccacagaagttgggctagctacggcttgagagata

Query-      9616 GTTGTACGGTGAATTGAAGTTGCAGATATGGGAACGGCTATATGATGGAAGTAAAGGCA
PIR:T04448      353 C Y G E L K L Q I W E R L Y D G S K G K
GSDB:S:495-    383 gttgttacggtgaattgaagttgcagatatgggaacggctatatgatggaagtaaaggca
```

Figure 31D

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Query- 9556 AGGTATGTATGCTAATGTGATCCCAATCCCTGTAGTTAAAAGTCTTAACAAATCCTAAGGC
 PIR:T04448 373 L K V L T N P K A
 GSDB:S:495- 323 ag
 PIR:T04448 Exon 9704 9555 Confidence: 100 100
 GSDB:S:495- Exon 9704 9555 Confidence: 98 100

Query- 9496 AGTGAAAGAAGATTATGAACGTTTGTATGGTTAACAATGATGCAGGTGATATTAGAGAC
 PIR:T04448 382 V K E D Y E R L L W L T M M Q V I L E T
 GSDB:S:495- 321 gtgatattagagac

Query- 9436 AAAGAGCTCAATGGCAGCAGTGGAGATAGGAGGAGGACCGTGGTTGGGACATGGAAAGG
 PIR:T04448 402 K S S M A A V E I G G G P W F G T W K G
 GSDB:S:495- 307 aaagagctcaatggcaNcagtgaggataggaggaggaccgtggttgggacatggaaagg

Query- 9376 AGATACGAGCAACACGCCCGAGCTACTAAAACAGGCTCTTCAGGTCCCATTGGATCTTGA
 PIR:T04448 422 D T S N T P E L L K Q A L Q V P L D L E
 GSDB:S:495- 247 agatacgagcaacacgccccgagctactaaaacaggctcttcaggtcccattggatcttga

Query- 9316 AAGCGCCTTAGGTTTGGTCCCTTTCTTCAAGCCACCGGGTCTG TAA
 (stop)
 PIR:T04448 442 S A L G L V P F F K P P G L
 GSDB:S:495- 187 aagcgccttaggtttgtccctttcttcaagccaccgggtctgtaacattgatgagtggt
 PIR:T04448 Exon 9522 9274 Confidence: 100 100

Query- 9256
 PIR:T04448 456
 GSDB:S:495- 127 ttgtttgttgatagagaccatgtgatgaatgaagccttagtcatgtcattgctagcttc

Query- 9196 ACTATTATGTATGTATGATTTAGTTCGTTTCGGTCCTTGTGGTAAATGATACGGGCCAGT
 GSDB:S:495- 67 actattatgtatgtatgatttagttcgttcggtccttgtggtaaatgatacgggccagt

Query- 9136 GTAAAGTCTAGTTCAATAAAAGCCTTGAGTCGCATAATTTCAAATTCAAATTGCATC
 GSDB:S:495- 7 gtaaagt
 GSDB:S:495- Exon 9450 9130 Confidence: 98 100

ATCEA4C37145_1 3063693/emb|CAA18584.1| 4.0e-43 (AL022537) putative protein
 [Arabidopsis thaliana]
 PIR:T04448 sPIR-T04448 shypothetical protein F4D11.30 - Arabidopsis thaliana;
 g3063693|emb|CAA18584.1 (AL022537) putative protein [Arabidopsis thaliana]_F4D11.30
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Figure 31E

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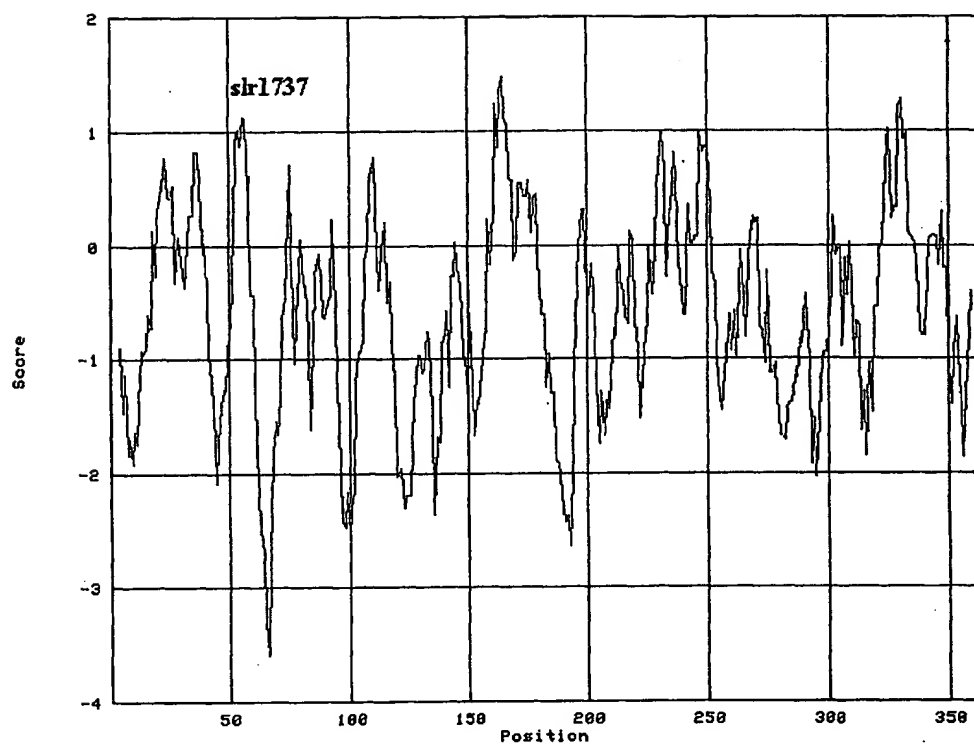


Figure 32

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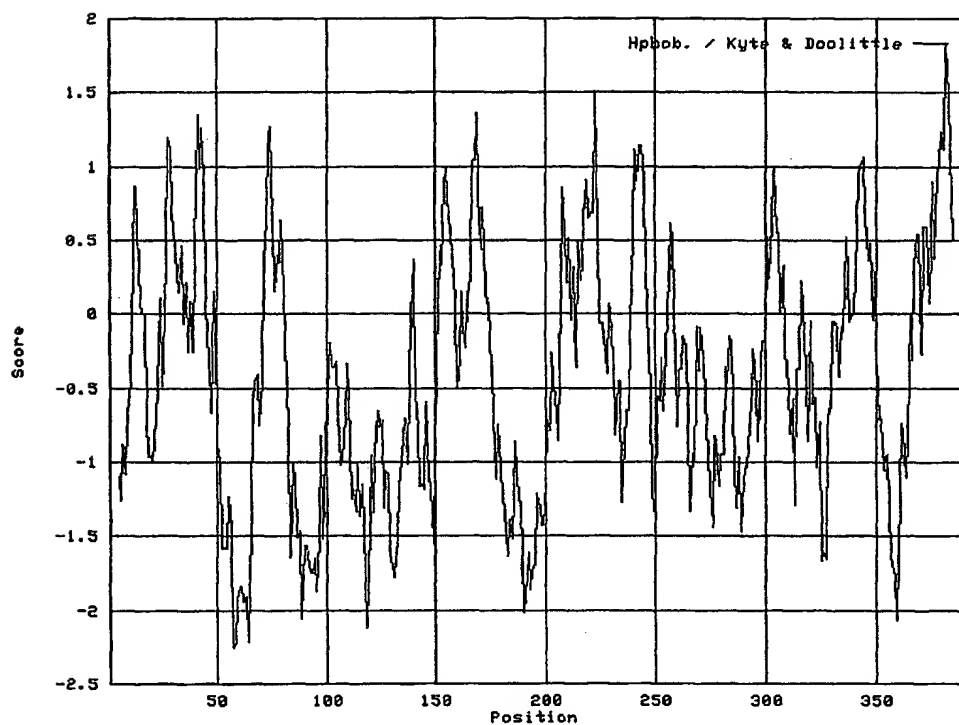


Figure 33

39/40

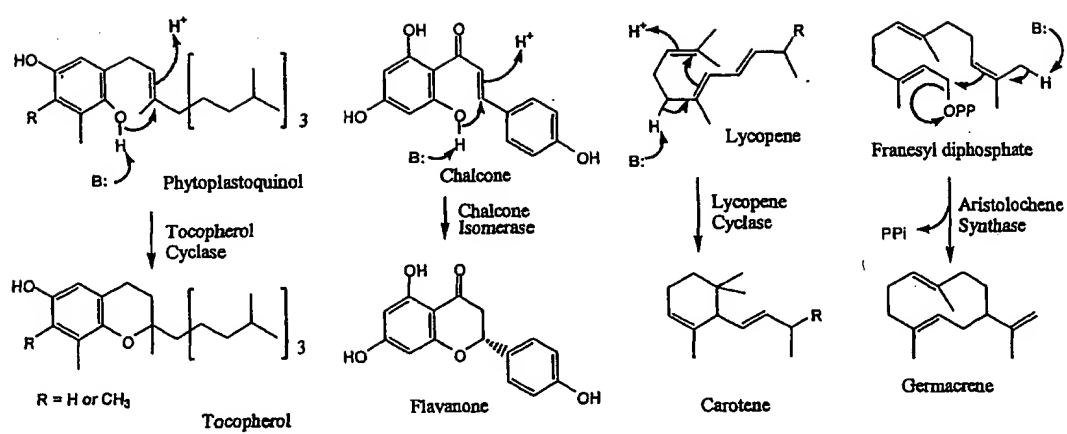


Figure 34

40/40

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slr1737_SYNSP_S74814_	-----DWGLTEENLSKKT-----VPF-----
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Figure 35

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 Chen, Ridong
 Valentin, Henry
 Huang Wong, Yun-Hua
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Glu Ser Thr Asp Ile Val Thr Ser Glu Leu Arg Val Arg Gln Arg Gly

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 Lys Val Lys Ser Gln Thr Pro Asp Lys Ala Pro Ala Gly Gly Ser Ser
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 Ala Tyr Ser Ile Asn Val Pro Leu Leu Arg Trp Lys Arg Phe Ala Val
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 115 120 125
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30

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 gatattcattg tottgactac tttgtacagc atagctgggc tagggattgc tattgtaaat 180
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Pro Leu Leu Gly Thr Ile Ala Leu Gly Thr Leu Ala Thr Ser Gly Leu
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    65             70             75             80
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    180            185            190
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Phe Gly Gln Tyr Val Gly Glu Ala Val Gly Ile Phe Phe Ala Leu Thr
    210            215            220
Ile Gly Cys Leu Phe Tyr Leu Gly Met Ile Leu Met Leu Asn Pro Leu
    225            230            235            240
35  Tyr Trp Leu Ser Leu Ala Ile Ala Ile Val Gly Trp Val Ile Gln Tyr
    245            250            255
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24

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 5 260 265 270
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 25 Gly Val Val Cys Gly Ala Ala Ser Ser Gly Gly Tyr Ile Trp Ser Val
 50 55 60
 Glu Asp Phe Leu Lys Ala Leu Thr Cys Met Leu Leu Ser Gly Pro Leu
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 30 85 90 95
 Asp Ala Ile Asn Glu Pro Tyr Arg Pro Ile Pro Ser Gly Ala Ile Ser
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 Val Pro Gln Val Val Thr Gln Ile Leu Ile Leu Leu Val Ala Gly Ile
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 130 135 140
 Met Met Val Leu Thr Leu Gly Gly Ala Phe Val Ala Tyr Ile Tyr Ser
 145 150 155 160
 Ala Pro Pro Leu Lys Leu Lys Gln Asn Gly Trp Leu Gly Asn Tyr Ala

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 5 195 200 205
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 35 Trp His Gly Asp Val Phe Thr Ile Phe Leu Leu Ser Ala Ile Ala Ile
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 65 70 75 80
 Ile Asp Val Arg Lys Ala His Ser Val Val Asn Leu Thr Gly Asn Arg

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	Phe Ser Trp Asn Leu Leu Thr Pro Ser Val Phe Val Gly Ile Ser Thr		
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	Ala Ile Ile Leu Phe Cys Ser His Phe His Gln Val Glu Asp Asp Leu		
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	Ala Ala Gly Lys Lys Ser Pro Ile Val Arg Leu Gly Thr Lys Leu Gly		
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	225	230	235
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	245	250	255
	Ala Ser Leu Pro Trp Ala Val Gln Leu Ile Arg His Val Gly Gln Tyr		
	260	265	270
	His Asp Gln Pro Glu Gln Val Ser Asn Cys Lys Phe Ile Ala Val Asn		
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 35 40 45
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 50 55 60
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 Pro Leu Ala Asn Gly Asp Phe Ser Ile Ala Gln Gly Arg Trp Ile Val
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 Gly Leu Cys Gly Val Ala Ser Leu Ala Ile Ala Trp Gly Leu Gly Leu
 100 105 110
 35 Trp Leu Gly Leu Thr Val Gly Ile Ser Leu Ile Ile Gly Thr Ala Tyr
 115 120 125
 Ser Val Pro Pro Val Arg Leu Lys Arg Phe Ser Leu Leu Ala Ala Leu
 130 135 140
 Cys Ile Leu Thr Val Arg Gly Ile Val Val Asn Leu Gly Leu Phe Leu

145 150 155 160
 Phe Phe Arg Ile Gly Leu Gly Tyr Pro Pro Thr Leu Ile Thr Pro Ile
 165 170 175
 Trp Val Leu Thr Leu Phe Ile Leu Val Phe Thr Val Ala Ile Ala Ile
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 Phe Lys Asp Val Pro Asp Met Glu Gly Asp Arg Gln Phe Lys Ile Gln
 195 200 205
 Thr Leu Thr Leu Gln Ile Gly Lys Gln Asn Val Phe Arg Gly Thr Leu
 210 215 220
 10 Ile Leu Leu Thr Gly Cys Tyr Leu Ala Met Ala Ile Trp Gly Leu Trp
 225 230 235 240
 Ala Ala Met Pro Leu Asn Thr Ala Phe Leu Ile Val Ser His Leu Cys
 245 250 255
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 15 260 265 270
 Lys Thr Glu Ile Ala Ser Phe Tyr Gln Phe Ile Trp Lys Leu Phe Phe
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	Glu Asn Gln Glu Asp Gln Leu Val Trp Arg Thr Phe Pro Ser Val Lys	
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Tyr Glu Phe Asp His Ala Leu Val Tyr Ala Glu Lys Asn Trp Gly His
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 210 215 220
 5 His Pro Gly Leu Ser Val Thr Ala Ala Gly Gly Glu Arg Ile Val Leu
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 Gly Arg Pro Glu Glu Val Ala Leu Ile Gly Leu His His Gln Gly Asn
 245 250 255
 Phe Tyr Glu Phe Gly Pro Gly His Gly Thr Val Thr Trp Gln Val Ala
 10 260 265 270
 Pro Trp Gly Arg Trp Gln Leu Lys Ala Ser Asn Asp Arg Tyr Trp Val
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 290 295 300
 15 Thr Ala Gln Gly Leu Gln Leu Asn Cys Arg Asp Thr Thr Arg Gly Tyr
 305 310 315 320
 Leu Tyr Leu Gln Leu Gly Ser Val Gly His Gly Leu Ile Val Gln Gly
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 cctctgtttt ttgtgggatt attagaggct gtgggtgctg cattgtttat gaatgtttac 480
 45 attgtaggtc tgaatcaatt atttgacata gaaatagaca aggtcaataa acctgatctt 540
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 5 <213> CANOLA

<400> 103
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 caaaagacgt gatgaaagcc acaggtgcac tcactgaaa cgaaagtcca agagcagctc 540
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 30 cggacgcgtg ggtcgac 677

<210> 104
 <211> 1431
 35 <212> DNA
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 45 accatcagaa attcttcgaa tggaaatcct cctattgtag gatatcacat cggtcattaa 240
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<210> 105

<211> 1870

40 <212> DNA

<213> CORN

<400> 105

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 50 ccgtgcggcc cggcgggcc cggcgcgag atcattttct accaccatgt tgttcatac 240
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<212> DNA

<213> CORN

<400> 106

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<210> 107

<211> 362

30 <212> DNA

<213> COTTON

<400> 107

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<210> 108

50 <211> 575

<212> DNA

<213> TOMATO

<400> 108

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 tgttgctgcc ctattcatga acatttacat agttggttta aaccagttgt ctgacatcga 480
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<210> 109

<211> 1663

<212> DNA

<213> ARABIDOPSIS

30

<400> 109

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<210> 110

<211> 488

<212> PRT

40 <213> ARABIDOPSIS

<400> 110

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 Phe Glu Leu Ser Arg Pro Val Ser Pro Leu Thr Arg Ser Leu Val Pro
 20 25 30
 50 Phe Arg Ser Thr Lys Leu Val Pro Arg Ser Ile Ser Arg Val Ser Ala
 35 40 45
 Ser Ile Ser Thr Pro Asn Ser Glu Thr Asp Lys Ile Ser Val Lys Pro

	50		55		60
	Val Tyr Val Pro Thr Ser	Pro Asn Arg Glu Leu Arg Thr Pro His Ser			
	65	70	75	80	
5	Gly Tyr His Phe Asp Gly Thr Pro Arg Lys Phe Phe Glu Gly Trp Tyr				
		85	90	95	
10	Phe Arg Val Ser Ile Pro Glu Lys Arg Glu Ser Phe Cys Phe Met Tyr				
		100	105	110	
	Ser Val Glu Asn Pro Ala Phe Arg Gln Ser Leu Ser Pro Leu Glu Val				
		115	120	125	
15	Ala Leu Tyr Gly Pro Arg Phe Thr Gly Val Gly Ala Gln Ile Leu Gly				
		130	135	140	
	Ala Asn Asp Lys Tyr Leu Cys Gln Tyr Glu Gln Asp Ser His Asn Phe				
		145	150	155	160
20	Trp Gly Asp Arg His Glu Leu Val Leu Gly Asn Thr Phe Ser Ala Val				
		165	170	175	
	Pro Gly Ala Lys Ala Pro Asn Lys Glu Val Pro Pro Glu Glu Phe Asn				
		180	185	190	
25	Arg Arg Val Ser Glu Gly Phe Gln Ala Thr Pro Phe Trp His Gln Gly				
		195	200	205	
30	His Ile Cys Asp Asp Gly Arg Thr Asp Tyr Ala Glu Thr Val Lys Ser				
		210	215	220	
	Ala Arg Trp Glu Tyr Ser Thr Arg Pro Val Tyr Gly Trp Gly Asp Val				
		225	230	235	240
35	Gly Ala Lys Gln Lys Ser Thr Ala Gly Trp Pro Ala Ala Phe Pro Val				
		245	250	255	
	Phe Glu Pro His Trp Gln Ile Cys Met Ala Gly Gly Leu Ser Thr Gly				
		260	265	270	
40	Trp Ile Glu Trp Gly Gly Glu Arg Phe Glu Phe Arg Asp Ala Pro Ser				
		275	280	285	
45	Tyr Ser Glu Lys Asn Trp Gly Gly Gly Phe Pro Arg Lys Trp Phe Trp				
		290	295	300	
	Val Gln Cys Asn Val Phe Glu Gly Ala Thr Gly Glu Val Ala Leu Thr				
		305	310	315	320
50	Ala Gly Gly Gly Leu Arg Gln Leu Pro Gly Leu Thr Glu Thr Tyr Glu				
		325	330	335	
	Asn Ala Ala Leu Val Cys Val His Tyr Asp Gly Lys Met Tyr Glu Phe				
		340	345	350	
55	Val Pro Trp Asn Gly Val Val Arg Trp Glu Met Ser Pro Trp Gly Tyr				
		355	360	365	

Trp Tyr Ile Thr Ala Glu Asn Glu Asn His Val Val Glu Leu Glu Ala
 370 375 380

5 Arg Thr Asn Glu Ala Gly Thr Pro Leu Arg Ala Pro Thr Thr Glu Val
 385 390 395 400

Gly Leu Ala Thr Ala Cys Arg Asp Ser Cys Tyr Gly Glu Leu Lys Leu
 405 410 415

10 Gln Ile Trp Glu Arg Leu Tyr Asp Gly Ser Lys Gly Lys Val Ile Leu
 420 425 430

15 Glu Thr Lys Ser Ser Met Ala Ala Val Glu Ile Gly Gly Gly Pro Trp
 435 440 445

Phe Gly Thr Trp Lys Gly Asp Thr Ser Asn Thr Pro Glu Leu Leu Lys
 450 455 460

20 Gln Ala Leu Gln Val Pro Leu Asp Leu Glu Ser Ala Leu Gly Leu Val
 465 470 475 480

Pro Phe Phe Lys Pro Pro Gly Leu
 485

25